

Exhibit ①

Amended Claims

1. A transgenic non-human mammal containing an exogenous DNA sequence stably integrated in its genome, wherein said exogenous DNA sequence comprises the 5' 4.2 kb *Sau3A* - *Kpn1* promoter of the mouse whey acidic protein gene, operably linked to a DNA sequence encoding protein C and a signal peptide, wherein said whey acidic protein promoter is specifically active in mammary cells and said signal peptide is effective in directing the secretion of said protein C into the milk of said transgenic mammal, wherein the activated form of said secreted protein C has an enzymatic activity of at least 50% as plasma-derived protein C, and wherein said transgenic mammal is selected from the group consisting of mice, rats, rabbits, pigs, sheep, goats and cows.

2. The transgenic non-human mammal of claim 1, wherein said protein C is human protein C, and wherein said DNA sequence encoding protein C further comprises regulatory elements located in the non-coding regions of the human protein C gene, wherein said regulatory elements are the AUG start codon, donor and acceptor splice signals, the secretion peptide, translation termination signal, transcription termination signal, and polyadenylation signal.

3. The transgenic non-human mammal of claim 1, wherein said DNA sequence encoding human protein C comprises the human protein C gene from 21 basepairs upstream of the protein C start codon to the *NheI* site in the 3' end of the protein C gene.

4. The transgenic non-human mammal of claim 1, wherein said exogenous DNA sequence comprises a DNA sequence comprising the 5' 4.2 kb *Sau3A* - *Kpn1* promoter fragment of the mouse whey acidic protein promoter ligated directly or by a linker to a

fragment of the human protein C gene beginning 21 basepairs upstream of the protein C start codon and ending at the *NheI* site in the 3' end of the protein C gene.

6. A process for the production of protein C, comprising the steps of:

(A) providing a non-human transgenic mammal characterized by an exogenous DNA sequence stably integrated in its genome, wherein said exogenous DNA sequence comprises the 5' 4.2 kb *Sau3A* - *Kpn1* promoter of the mouse whey acidic protein gene, operably linked to a DNA sequence encoding protein C and a signal peptide, said promoter being specifically active in mammary cells and said signal peptide being effective in directing the secretion of said protein C into the milk of said transgenic mammal, and wherein said transgenic mammal is selected from the group consisting of mice, rats, rabbits, pigs, sheep, goats and cows;

(B) producing milk from said transgenic mammal, wherein said milk contains said secreted protein C, and wherein the activated form of said secreted protein C has an enzymatic activity of at least 50% as plasma-derived protein C;

(C) collecting said milk; and

(D) isolating said protein C from said milk.

7. The process of claim 6, wherein said protein C is human protein C, and wherein said DNA sequence encoding protein C further comprises regulatory elements located in the non-coding regions of the human protein C gene, wherein said regulatory elements are the AUG start codon, donor and acceptor splice signals, the secretion peptide, translation termination signal, transcription termination signal, and polyadenylation signal.

8. The process of claim 6, wherein said DNA sequence encoding human protein C comprises the human protein C gene from

21 basepairs upstream of the protein C start codon to the *NheI* site in the 3' end of the protein C gene.

9. The process of claim 6, wherein said exogenous DNA comprises a DNA sequence comprising the 5' 4.2 kb *Sau3A* - *Kpn1* promoter fragment of the mouse whey acidic protein promoter ligated directly or by a linker to a fragment of the human protein C gene beginning 21 basepairs upstream of the protein C start codon and ending at the *NheI* site in the 3' end of the protein C gene.

11. A process for producing non-human transgenic mammals, comprising the steps of (A) providing a mixture containing a double-stranded DNA; (B) subjecting said mixture to anion-exchange high performance liquid chromatography to obtain purified double-stranded DNA; and thereafter (C) microinjecting an aqueous buffer solution containing said purified double-stranded DNA into an animal embryo, wherein said double-stranded DNA is selected from the group consisting of a double-stranded DNA comprising the 5' 4.2 kb *Sau3A* - *Kpn1* promoter fragment of the mouse whey acidic protein promoter, a double-stranded DNA comprising a fragment of the human protein C gene beginning 21 basepairs upstream of the protein C start codon and ending at the *NheI* site in the 3' end of the protein C gene, and a double-stranded DNA comprising a DNA sequence comprising the 5' 4.2 kb *Sau3A* - *Kpn1* promoter fragment of the mouse whey acidic protein promoter ligated directly or by a linker to a fragment of the human protein C gene beginning 21 basepairs upstream of the protein C start codon and ending at the *NheI* site in the 3' end of the protein C gene, wherein the activated form of protein C encoded by said double-stranded DNA has an enzymatic activity of at least 50% as plasma-derived protein C, and wherein said transgenic mammal is selected from the group consisting of mice, rats, rabbits, pigs, sheep, goats and cows.

12. A process for the production of a polypeptide in the milk of a transgenic non-human mammal, comprising the steps of:

(A) providing a non-human transgenic mammal characterized by an exogenous DNA sequence stably integrated in its genome, wherein said exogenous DNA sequence comprises the 5' 4.2 kb *Sau3A* - *Kpn1* promoter of the mouse whey acidic protein gene, operably linked to a DNA sequence encoding said polypeptide and a signal peptide, said promoter being specifically active in mammary cells and said signal peptide being effective in directing the secretion of said polypeptide into the milk of said transgenic mammal;

(B) producing milk from said transgenic mammal, wherein said milk contains said secreted polypeptide;

(C) collecting said milk; and

(D) isolating said polypeptide from said milk.

14. A transgenic non-human mammal containing an exogenous DNA sequence stably integrated in its genome, wherein said exogenous DNA sequence comprises the 5' 4.2 kb *Sau3A* - *Kpn1* promoter fragment of the mouse whey acidic protein promoter, operably linked to a DNA encoding a polypeptide whereby said polypeptide is expressed specifically in mammary cells of said transgenic mammal and said polypeptide comprises a signal peptide, said signal peptide being effective in directing the secretion of said polypeptide into the milk of said mammal.

16. An isolated DNA molecule which regulates the expression of a heterologous gene, wherein said DNA molecule consists of the 5' 4.2 kb *Sau3A* - *Kpn1* promoter of the mouse whey acidic protein gene.

17. The process of claim 12, wherein said transgenic non-human mammal is selected from the group consisting of mice, rats, rabbits, pigs, sheep, goats and cows.

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18. The process of claim 17, wherein said transgenic non-human mammal is sheep.

19. The process of claim 17, wherein said transgenic non-human mammal is a goat.

20. The process of claim 17, wherein said transgenic non-human mammal is a cow.

21. The transgenic non-human mammal of claim 14, wherein said transgenic non-human mammal is selected from the group consisting of mice, rats, rabbits, pigs, sheep, goats and cows.

22. The process of claim 21, wherein said transgenic non-human mammal is sheep.

23. The process of claim 21, wherein said transgenic non-human mammal is a goat.

24. The process of claim 21, wherein said transgenic non-human mammal is a cow.

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(54) Title: PRODUCTION OF RECOMBINANT POLYPEPTIDES BY BOVINE SPECIES AND TRANSGENIC METHODS (57) Abstract <p>A transgene for producing recombinant polypeptides in the milk of transgenic bovine species comprises at least one expression regulation sequence, a secretory DNA sequence encoding a secretory signal which is functional in mammary secretory cells of the bovine species and a recombinant DNA sequence encoding the recombinant polypeptides. A method for producing transgenic bovine species comprises introducing the above transgene into an embryonal target cell of a bovine species, transplanting the transgenic embryonal target cell formed thereby into a recipient bovine parent and identifying at least one female offspring which is capable of producing the recombinant polypeptide in its milk. The invention also includes transgenic bovine species capable of producing recombinant polypeptides in transgenic milk as well as the milk from such transgenic bovine species and food formulations containing one or more recombinant polypeptide. A method for producing transgenic non-human mammals having a desirable phenotype comprises first methylating a transgene followed by introduction into fertilized oocytes. The oocytes are then cultured to form preimplantation embryos. Thereafter, at least one cell is removed from each of the pre-implantation embryos and the DNA digested with a restriction endonuclease capable of cleaving the methylated transgene but incapable of cleaving the unmethylated form of the transgene.</p>		

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**PRODUCTION OF RECOMBINANT POLYPEPTIDES
BY BOVINE SPECIES AND TRANSGENIC METHODS**

Field of the Invention

The invention relates to the production of recombinant polypeptides by transgenic bovine species and to methods for producing transgenic non-human mammals having a
5 desired phenotype.

Background of the Invention

There is a plethora of literature relating to the expression of heterologous genes in lower organisms such as unicellular bacteria, yeast and filamentous fungi,
10 and in higher cell types such as mammalian cells. There are also numerous reports on the production of transgenic animals, most of which relate to the production of transgenic mice. See, e.g. U.S. Pat. No. 4,736,866 (transgenic mice containing activated
15 oncogene); Andres, A., et al. (1987) Proc. Natl. Acad. Sci. USA 84, 1299-1303 (HA-RAS oncogene under control of whey acid protein promoter); Schoenberger, C.A., et al. (1987) Experientia 43, 644 and (1988) EMBO J. 7. 169-175 (C-myc oncogene under control of whey acid
20 protein promoter); and Muller, W.J., et al. (1988) Cell 54, 105-115 (C-myc oncogene under control of the mouse mammary tumor virus promoter). Several laboratories have also reported the production of transgenic porcine species (Miller, K.F., et al. (1989), J. Endocrin., 120,
25 481-488 (expression of human or bovine growth hormone

gene in transgenic swine); Vize, P.D., et al. (1988), J. Cell Sci., 90, 295-300 (porcine growth hormone fusion gene in transgenic pigs); and Ebert, K. et al. (1988), Mol. Endocrin., 2, 277-283 (MMLV-rat somatotropin fusion gene in transgenic pigs)), transgenic sheep (Nancarrow, et al. (1987), Theriogenology, 27, 263 (transgenic sheep containing bovine growth hormone gene) Clark, A.J. et al. (1989) Bio/Technology 7, 487-482 and Simons, J., et al. (1988) Bio/Technology 6, 179-183 (human factor IX and α -1 antitrypsin CONA in ovine species), and rabbit (Hanover, S.V., et al. (1987), Deutsche Tierarztliche Wochenschrift, 94, 476-478 (production of transgenic rabbits by injection of uteroglobin-promoter-CAT fusion gene into fertilized rabbit oocytes). A number of reports have also suggested the production of transgenic cattle (Wagner, et al. (1984), Theriogenology, 21, 29-44) with one reporting some progress in microinjection techniques (Lohse, J.K., et al. (1985), Theriogenology, 23, 205). However, little, if any, success has been achieved in producing transgenic cows. Scientific articles which clearly demonstrate the actual production of a transgenic cow capable of producing a heterologous protein are presently unknown. This, despite the statements that one transgenic cow was produced in Canada which expressed human β -interferon (Van Brunt, J. (1988), Bio/Technology, 6, 1149-1155) and that transient expression of human α -fetoprotein in liver and blood was obtained on one occasion (Church, R.B. (1986), Biotechnology News Watch, 6 (15), 4). One reference reports that bovine papilloma virus was apparently integrated but not expressed in a transgenic cow (Roschlau et al. (1988) Arch. Tierz., Berlin 31, 3-8). A recent article has summarized the genetic engineering of livestock. (Pursel, V.G. et al. (1989), Science, 244, 1281-1288).

A number of laboratories have reported tissue-specific expression of DNA encoding various proteins in the mammary gland or the production of various proteins in the milk of transgenic mice and sheep. For example, Simmons, J.P., et al. (1987) Nature 328, 530-532 report the microinjection of a 16.2kb genomic fragment encoding β -lactoglobulin (BLG) including 4kb of 5' sequence, 4.9kb of the BLG transcription unit and 7.3kb of 3' flanking sequence into fertilized mouse eggs. According to these authors, the sheep BLG was expressed in mammary tissue and produced BLG in the milk of the transgenic mice at concentrations ranging from about 3.0 to about 23 mg/ml. When, however, cDNA encoding human factor IX or human α 1-antitrypsin was inserted into the 5' untranslated region of the BLG gene and microinjected into sheep (Simmons, J.P., et al. (1988) Bio/Technology 6, 179-183) the production of factor IX or α 1-antitrypsin was significantly reduced (25ng/ml for factor IX and 10mg/ml for α 1-antitrypsin; see Clark, A.J., et al. (1989) Bio/Technology 7, 487-492).

In a similar approach, a 14kb genomic clone containing the entire 7.5kb rat β -casein together with 3.5kb of 5' and 3.0kb of 3' flanking DNA was reportedly microinjected into fertilized mouse oocytes. Lee, et al. (1988) Nucl. Acids Res. 16 1027-1041. Yet, in this case, the level of expression of the rat β -transgene in the lactating mammary gland of transgenic mice was reported to be at a level of 0.01-1% of the endogenous mouse β -casein gene.

Human tissue plasminogen activator (t-PA) reportedly was produced in transgenic mouse milk at the levels between 0.2 and about 0.4 μ g/ml when a cDNA encoding a human t-PA with its endogenous secretion sequence was expressed under control of a 2.6kb 5' sequence of the murine whey acid protein gene. Gordon, K., et al. (1987)

- Bio/Technology 5, 1183-1187. Subsequent experiments using the same or similar construction reportedly produced t-PA in different mouse lines arranging from less than 20ng of t-PA per ml of milk to about 50 μ g/ml.
- 5 Pittius, C.W., et al. (1988) Proc. Natl. Acad. Sci. USA 85, 5874-5878.

U.S. Patent No. 4,873,316 issued October 10, 1989, discloses the use of 9kb of 5' sequence from the bovine α S1-casein gene including the casein signal peptide and
10 several casein codons fused to a mature t-PA sequence. The transgenic mice obtained with this construct reportedly produced about 0.2-0.5 μ g/ml of a t-PA fusion protein in their milk.

In addition, a number of patent publications purportedly
15 describe the production of specific proteins in the milk of transgenic mice and sheep. See, e.g. European Patent Publication No. 0 264 166 published April 20, 1988 (hepatitis B surface antigen and t-PA genes under control of the whey acid promoter protein for mammary
20 tissue specific expression in mice); PCT Publication No. W088/00239 published January 14, 1988 (tissue specific expression of a transgene encoding factor IX under control of a whey protein promoter in sheep); PCT Publication No. W088/01648 published March 10, 1988
25 (transgenic mouse having mammary secretory cells incorporating a recombinant expression system comprising a bovine α -lactalbumin gene fused to interleukin-2); European Pat. Pub. No. 0 279 582 published August 24, 1988 (tissue-specific expression of chloramphenicol
30 acetyltransferase under control of rat β -casein promoter in transgenic mice); and PCT Pub. No. W088/10118 published December 29, 1988 (transgenic mice and sheep containing transgene encoding bovine α S1-casein promoter and signal sequence fused to t-PA).

Given the state of the transgenic art, it is apparent that a need exists for methods which enable the efficient production of transgenic mammals, especially transgenic mammals other than transgenic mice.

- 5 Further, it is apparent that a need exists for methods for producing transgenic bovine species which are capable of producing recombinant polypeptides such as human milk proteins and human serum proteins in the milk of such transgenic mammals.
- 10 Accordingly, it is an object herein to provide methods for detecting the transgenesis of fertilized oocytes prior to implantation.

In addition, it is an object herein to provide transgenic bovine species which are capable of producing
15 recombinant polypeptides which are maintained intracellularly or are secreted extracellularly.

It is also an object herein to provide transgenic bovine species which are capable of producing recombinant polypeptides such as human milk proteins and human serum
20 proteins in the milk of such transgenic animals.

Further, it is an object herein to provide milk from a transgenic bovine species containing such recombinant polypeptides.

Still further, it is an object herein to provide food
25 formulations supplemented with recombinant polypeptides from such transgenic milk such as human infant formula supplemented with human lactoferrin.

Further, it is an object herein to provide transgenes which are capable of directing the production of

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recombinant polypeptides in the milk of transgenic bovine species.

The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by priority based on earlier filed applications.

Summary of the Invention

10 In accordance with the above objects, the invention includes transgenes for producing recombinant polypeptides in the milk of transgenic bovine species. The production of such transgenic bovine milk containing one or more recombinant polypeptides is desirable since
15 it provides a matrix wherein little or no purification is necessary for human consumption. The transgene comprises a secretory DNA sequence encoding a secretory signal sequence which is functional in mammary secretory cells of the bovine species of interest and a
20 recombinant DNA sequence encoding the recombinant polypeptide. These sequences are operably linked to form a secretory-recombinant DNA sequence. At least one expression regulation sequence, functional in the mammary secretory cells of the bovine species, is
25 operably linked to the secretory-recombinant DNA sequence. The transgene so constructed is capable of directing the expression of the secretory-recombinant DNA sequence in mammary secretory cells of bovine species containing the transgene. Such expression
30 produces a form of recombinant polypeptide which is secreted from the mammary secretory cells into the milk of the transgenic bovine species.

In addition, the invention includes methods for producing such transgenic bovine species. The method

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includes introducing the above transgene into an embryonal target cell of a bovine species, transplanting the transgenic embryonic target cell formed thereby into a recipient bovine parent and identifying at least one
5 female offspring which is capable of producing the recombinant polypeptide in its milk.

The invention also includes transgenic bovine species capable of producing recombinant polypeptides in the milk of lactating females of said species, the milk from
10 such transgenic bovine species containing such recombinant polypeptides and food formulations containing the transgenic milk in liquid or dried form, as well as food formulations supplemented with one or more recombinant polypeptides from such transgenic milk.

15 In addition to the foregoing, the invention includes transgenes and transgenic bovine species containing transgenes that are capable of producing a recombinant polypeptide. Such transgenes are similar to the aforementioned transgenes for milk secretion and are
20 characterized by having an expression regulation sequence which targets the expression of the DNA encoding the recombinant polypeptide to a particular cell or tissue type, e.g. expression of human serum albumin in the liver of a transgenic bovine species.
25 When the recombinant polypeptide is to be secreted from such targeted cells or tissues, a secretory DNA sequence encoding a secretory signal sequence functional in the particular targeted cell or tissue is operably linked to the recombinant DNA sequence encoding the recombinant
30 polypeptide, e.g. secretion of human serum albumin from bovine liver into the bovine circulatory system.

Further, the invention includes methods for producing transgenic non-human mammals having a desirable

phenotype. The method comprises first causing the methylation of a transgene capable of conferring the desirable phenotype when incorporated into the cells of a transgenic non-human animal, e.g., by transforming an appropriate bacterium, such as *E. coli* MM 294, with a plasmid containing the transgene. The methylated transgene is then excised and introduced into fertilized oocytes of the non-human animal to permit integration into the genome. The oocytes are then cultured to form pre-implantation embryos thereby replicating the genome of each of the fertilized oocytes. Thereafter, at least one cell is removed from each of the pre-implantation embryos and treated to release the DNA contained therein. Each of the released DNAs are then digested with a restriction endonuclease capable of cleaving the methylated transgene but incapable of cleaving the unmethylated form of the transgene formed after integration into and replication of the genomic DNA. Those pre-implantation embryos which have integrated the transgene contain DNA which is resistant to cleavage by the restriction endonuclease in the region containing the transgene. This resistance to digestion, which can be detected by electrophoresis of the digest after PCR amplification of the DNA and hybridization with a labelled probe for the transgene, facilitates the identification of successful transgenesis.

The invention also includes a method to produce a population of transgenic offspring having the same genotype. This method utilizes a specific embodiment of the above method for detecting early transgenesis. In this method, a methylated transgene is introduced into fertilized oocytes which are cultured to pre-implantation embryos. Thereafter, each pre-implantation embryo is divided to form first and second hemi-embryos. Each of the first hemi-embryos are then analyzed for transgenesis as described above. After identifying

successful transgenesis in at least one first hemi-embryo, the second untreated hemi-embryo which contains the integrated transgene, is cloned to form a multiplicity of clonal transgenic blastocysts or hemi-
5 blastocysts, each of which have the same genotype. The transgenic embryos are thereafter transplanted into one or more recipient female parents to produce a population of transgenic non-human mammals having the same genotype.

10 Brief Description of the Drawings

The accompanying drawings, which are incorporated in and form a part of the specification, illustrate embodiments of the present invention and, together with the description, serve to explain the principles of the
15 invention. In the drawings:

Fig. 1 depicts the DNA (Seq. ID No.: 1) and amino acid (Seq. ID No.: 2) sequence for a human lactoferrin clone derived from a human mammary cDNA library as described herein except that the sequence between nucleotides
20 1557-1791 and 2050-2119 corresponds to the previously published sequence (Rado et al. (1987) Blood 70, 989-993).

Fig. 2 depicts the complete DNA (Seq. ID No.: 3) and amino acid (Seq. ID No.: 4) sequence of human
25 lactoferrin including 5' and 3' untranslated sequence as well as the complete human lactoferrin signal sequence.

Fig. 3 is a restriction map of a clone of a 5'-flanking region of bovine α S1-casein gene.

30 Fig. 4 is a restriction map of a clone of a 3'-flanking region of bovine α S1-casein gene.

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Figs. 5A, 5B and 5C depict the construction of pSI3'5'CAT and pSI5'CAT.

Fig. 6 depicts pMH-1.

5 Figs. 7A through 7F depict the construction of expression vectors containing sequences encoding human lactoferrin.

10 Fig. 8 depicts the genome of human serum albumin, the fragments used to generate transgenic mice contained in this genomic DNA and the identification of the fragment sizes which would be obtained upon the digestion of genomic DNA from a transgenic mouse with the restriction enzymes BstE-II and Nco-I or with Nco-I and Hindi-III.

15 Fig. 9 depicts an alternate pathway for the construction of a transgene of the invention encoding human lactoferin.

Fig. 10 depicts the construction of a plasmid pPC containing a transgene encoding Protein C.

20 Fig. 11 depicts the DNA sequence for a hybrid intervening sequence used in a preferred embodiment of the invention. This hybrid sequence comprises a 5' portion from an intervening sequence of bovine α S1-casein and a 3' portion from an intervening sequence of an IgG intervening sequence. The juncture of the 5' and 3' portion is the HindIII site shown.

25 Detailed Description of the Invention

The "non-human mammals" of the invention comprise all non-human mammals capable of producing a "transgenic non-human mammal" having a "desirable phenotype". Such mammals include non-human primates, murine species, 30 bovine species, canine species, etc. Preferred non-

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human animals include bovine, porcine and ovine species, most preferably bovine species.

Desirable phenotypes for transgenic non-human mammals include, but are not limited to, the production of
5 recombinant polypeptides in the milk of female transgenic non-human mammals, the production of animal models for the study of disease, the production of animals with higher resistance to disease (e.g. diseases of the mammary gland such as mastitis) and the
10 production of recombinant polypeptides in the blood, urine or other suitable body fluid or tissue of the animal. In the preferred embodiments, transgenic bovine species are disclosed which are capable of producing recombinant human lactoferrin, human serum albumin and
15 human Protein C in the milk of lactating females or human serum albumin in the liver of the transgenic animal.

The transgenic non-human mammals of the invention are produced by introducing a "transgene" into an embryonal
20 target cell of the animal of choice. In one aspect of the invention, a transgene is a DNA sequence which is capable of producing a desirable phenotype when contained in the genome of cells of a transgenic non-human mammal. In specific embodiments, the transgene
25 comprises a "recombinant DNA sequence" encoding a "recombinant polypeptide". In such cases, the transgene is capable of being expressed to produce the recombinant polypeptide.

As used herein, a "recombinant polypeptide" (or the
30 recombinant DNA sequence encoding the same) is either a "heterologous polypeptide" or a "homologous polypeptide". Heterologous polypeptides are polypeptides which are not normally produced by the transgenic animal. Examples of heterologous

polypeptides include human milk proteins such as lactoferrin, lysozyme, secreted immunoglobulins, lactalbumin, bile salt-stimulated lipase, etc., human serum proteins such as albumin, immunoglobulins, Factor
5 VIII, Factor IX, protein C, etc. and industrial enzymes such as proteases, lipases, chitinases, and ligninases from procaryotic and eucaryotic sources. The recombinant DNA sequences include genomic and cDNA sequences encoding the recombinant polypeptide.

10 When recombinant DNA sequences encoding a heterologous polypeptide are used, the transgene may be integrated in a random manner into the genome of the species used for transgenesis. As disclosed in the Examples, transgenes encoding human lactoferrin, human serum
15 albumin and human Protein C in conjunction with a α S1-casein secretory signal sequence under control of α S1-casein expression regulation sequences are designed to produce and secrete these heterologous polypeptides from the mammary gland of a lactating transgenic mammal into
20 its milk.

As used herein, a homologous polypeptide is one which is endogenous to the particular transgenic species. Examples of endogenous polypeptides from bovine species include bovine milk proteins such as α S1, α S2, β - and
25 κ -casein, β -lactoglobulin, lactoferrin, lysozyme, cholesterol hydrolase, serum proteins such as serum albumin and proteinaceous hormones such as growth hormones. When recombinant DNA sequences encoding a homologous polypeptide are used, the transgene is
30 preferably integrated in a random manner into the genome of the species used for transgenesis. Such random integration results in a transgenic animal which contains not only the transgene encoding the endogenous polypeptide but also the corresponding endogenous
35 genomic DNA sequence. Accordingly, such transgenic non-

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human mammals are readily characterized by an increase in the copy number of genes encoding the endogenous polypeptide. Further, the transgene will generally be located at a position which is different from the
5 endogenous gene.

When DNA encoding a homologous polypeptide is expressed, for example, in bovine species, the transgenic animal is characterized by an increase in the amount of the homologous polypeptide in either the endogenous tissue
10 or fluid in which it is normally found and/or by its presence in a tissue and/or body fluid which either does not normally contain the homologous polypeptide or produces it at significantly lower levels.

Thus, for example, bovine cholesterol hydrolase is
15 normally present in the colostrum for about the first 15-20 days of lactation. This naturally occurring endogenous polypeptide increases calf weight. This protein, however, is also a homologous polypeptide when, for example, its expression in mammary secretory cells
20 is placed under the control of expression regulation sequences, such as those obtained from bovine casein genes, which facilitate the expression of the homologous polypeptide beyond the lactation period that it is normally present. Thus, according to one aspect of the
25 invention, bovine cholesterol hydrolase expression is maintained in transgenic bovine milk by placing the expression of cholesterol hydrolase recombinant DNA (either cDNA or genomic) under the control of bovine α S1-casein expression regulation sequences. When a
30 genomic recombinant DNA is used, it is engineered such that it has appropriate restriction sites (e.g. ClaI and SalI) at the 5' and 3' end of the structural gene such that it is capable of being inserted into an appropriate transgene genomic cassette (e.g. p-16 Kb, CS which is
35 described in Example 15). Alternatively, a recombinant

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DNA encoding bovine cholesterol hydrolase derived from cDNA may be placed under control of bovine α S1-casein expression regulation sequence by substituting the human lactoferrin sequences in a plasmid such as p16, 8HLF3 (containing a hybrid intervening sequence) or p16, 8HLF4 (containing a homologous α S1-casein intervening sequence). When these particular plasmids are used, the cDNA clone is engineered such that it has appropriate ClaI and SalI restriction sites at the ends of the recombinant DNA.

By way of further example, bovine lactoferrin is normally present in only trace amounts in cow's milk. When, however, bovine lactoferrin is expressed under control of other regulatory sequences, for example, obtained from an α S1-casein gene, higher amounts of lactoferrin in the milk of transgenic bovine species are obtained. In another example, a transgene comprising DNA encoding homologous bovine growth hormone is incorporated into the bovine genome to confer superior growth characteristics to the transgenic animal. In other instances, homologous polypeptides include, for example, a polypeptide which normally is maintained intracellularly in a particular species but which is secreted into the milk or other extracellular compartment of the transgenic species, such as the circulatory system.

Each of the heterologous or homologous polypeptides are characterized by specific amino acid and nucleic acid sequences. It is to be understood, however, that such sequences include naturally occurring allelic variations thereof and variants produced by recombinant methods wherein such nucleic acid and polypeptide sequences have been modified by the substitution, insertion and/or deletion of one or more nucleotides in such nucleic acids to cause the substitution, insertion or deletion

of one or more amino acid residues in the recombinant polypeptide.

When expression of the DNA of the transgene is necessary to generate a desired phenotype, e.g. to produce a recombinant polypeptide, the transgene typically includes at least a 5' and preferably additional 3' "expression regulation sequences" each operably linked to a recombinant or secretory-recombinant DNA as defined hereinafter. Such expression regulation sequences in addition to controlling transcription also contribute to RNA stability and processing, at least to the extent they are also transcribed.

Such expression regulation sequences are chosen to produce tissue-specific or cell type-specific expression of the recombinant or secretory-recombinant DNA. Once a tissue or cell type is chosen for expression, 5' and optional 3' expression regulation sequences are chosen. Generally, such expression regulation sequences are derived from genes that are expressed primarily in the tissue or cell type chosen. Preferably, the genes from which these expression regulation sequences are obtained are expressed substantially only in the tissue or cell type chosen, although secondary expression in other tissue and/or cell types is acceptable if expression of the recombinant DNA in the transgene in such tissue or cell type is not detrimental to the transgenic animal. Particularly preferred expression regulation sequences are those endogenous to the species of animal to be manipulated. However, expression regulation sequences from other species such as those from human genes may also be used. In some instances, the expression regulation sequences and the recombinant DNA sequences (either genomic or cDNA) are from the same species, e.g., each from bovine species or from a human source. In such cases, the expression regulation sequence and

the recombinant DNA sequence are homologous to each other. Alternatively, the expression regulation sequences and recombinant DNA sequences (either cDNA or genomic) are obtained from different species, e.g., an expression regulation sequence from bovine species and a recombinant DNA sequence from a human source). In such cases, the expression regulation and recombinant DNA sequence are heterologous to each other. The following defines expression regulation sequences from endogenous genes. Such definitions are also applicable to expression regulation sequences from non-endogenous, heterologous genes.

In general, the 5' expression regulation sequence includes the transcribed portion of the endogenous gene upstream from the translation initiation sequence (the 5' untranslated region or 5' UTR) and those flanking sequences upstream therefrom which comprise a functional promoter. As used herein, a "functional promoter" includes those necessary untranscribed DNA sequences which direct the binding of RNA polymerase to the endogenous gene to promote transcription. Such sequences typically comprise a TATA sequence or box located generally about 25 to 30 nucleotides from the transcription initiation site. The TATA box is also sometimes referred to the proximal signal. In many instances, the promoter further comprises one or more distal signals located upstream from the proximal signal (TATA box) which are necessary to initiate transcription. Such promoter sequences are generally contained within the first 100 to 200 nucleotides located upstream from the transcription initiation site, but may extend up to 500 to 600 nucleotides from the transcription initiation site. Such sequences are either readily apparent to those skilled in the art or readily identifiable by standard methods. Such promoter sequences alone or in combination with the 5'

untranslated region are referred to herein as "proximal 5' expression regulation sequences".

In addition to such proximal 5' expression regulation sequences, it is preferred that additional 5' flanking sequences (referred to herein as "distal 5' expression regulation sequences") also be included in the transgene. Such distal 5' expression regulation sequences are believed to contain one or more enhancer and/or other sequences which facilitate expression of the endogenous gene and as a consequence facilitate the expression of the recombinant or secretory-recombinant DNA sequence operably linked to the distal and proximal 5' expression regulation sequences. The amount of distal 5' expression regulation sequence depends upon the endogenous gene from which the expression regulation sequences are derived. In general, however, such sequences comprise 5' flanking regions of approximately 1kb, more preferably 16kb and most preferably about 30kb of 5' flanking sequence. The determination of the optimal amount of distal 5' expression regulation sequence used from any particular endogenous gene is readily determined by varying the amount of distal 5' expression regulation sequence to obtain maximal expression. In general, the distal 5' expression regulation sequence will not be so large as to extend into an adjacent gene and will not include DNA sequences which adversely effect the level of transgene expression.

In addition, it is preferred that 3' expression regulation sequences also be included to supplement tissue or cell-type specific expression. Such 3' expression regulation sequences include 3' proximal and 3' distal expression regulation sequences from an appropriate endogenous gene. The 3' proximal expression regulation sequences include transcribed but

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untranslated DNA positioned downstream from the translation stop signal in the recombinant DNA sequence (also referred to as the 3' untranslated region or 3' UTR). Such sequences generally terminate at a polyadenylation sequence (either from the endogenous gene or from other sources such as SV40) and sequences that may affect RNA stability. Generally, 3' UTR's comprise about 100 to 500 nucleotides downstream from the translation stop signal in the gene from which the 3' regulation sequence is derived. Distal 3' expression regulation sequences include flanking DNA sequences downstream from the proximal 3' expression regulation sequence. Some of these distal sequences are transcribed, but do not form part of the mRNA while other sequences in this distal 3' expression regulation sequence are not transcribed at all. Such distal 3' expression regulation sequences are believed to contain enhancer and/or other sequences which enhance expression. Such sequences are believed to be necessary for efficient polydenylation and contain transcription termination sequences. Preferably, such sequences comprise about 2kb, more preferably 8kb and most preferably about 15kb of 3' flanking sequence.

Although the use of both 5' and 3' expression regulation sequences are preferred, in some embodiments of the invention, endogenous 3' regulation sequences are not used. In such cases, the 3' proximal expression regulation sequences normally associated with the genomic DNA encoded by the recombinant DNA sequence are used to direct polyadenylation. In addition, distal 3' regulation sequences from the genomic DNA encoding the recombinant polypeptide may also be employed preferably in the same amounts as set forth for endogenous 3' expression regulation sequences. In such cases, it is to be understood that the recombinant polypeptide encoded by the transgene may comprise either genomic DNA

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or a double stranded DNA derived from cDNA. As with the 5' expression regulation sequences, the optimal amount of 3' expression regulation sequence may be readily determined by varying the amount of 3' flanking sequence to obtain maximal expression of the recombinant polypeptide. In general, the distal 3' regulation sequence, be it from an endogenous gene or a heterologous gene, will not extend into the adjacent gene from which is derived and will exclude any sequences which adversely effect the level of transgene expression.

Examples of expression regulation sequences are provided in Table I.

TABLE 1

<u>Expression Regulation Sequence</u>	<u>Tissue Specificity</u>	<u>Animal Species</u>
16kb of bovine α S1 casein 5' to structural gene and 8kb 3' to structural gene	Mammary secretory cells	bovine
\approx 15kb 5' to albumin gene	Liver	murine
\approx 15kb 5' to α -actin gene	Muscle	murine
\approx 15kb upstream of protamine gene	Spermatids	murine

In addition to the 5' and 3' expression regulation sequences and the recombinant DNA (either genomic or derived from cDNA) the transgenes of the invention preferably also comprise a "recombinant intervening sequence" which interrupts the transcribed but untranslated 5' region of the transgene. Such intervening sequences can be derived, for example, from bovine α S1-casein and from human lactoferrin. Such

sequences as used herein are "homologous recombinant intervening sequences" in that the 5' and 3' RNA splice signals in such recombinant intervening sequences are those normally found in an intervening sequence from an endogenous or heterologous gene. Recombinant intervening sequences may, however, also comprise a "hybrid intervening sequence". Such hybrid intervening sequences comprise a 5' RNA splice signal and 3' RNA splice signal from intervening sequences from different sources. In some aspects of the invention, such hybrid intervening sequences comprise at least one "permissive RNA splice sequence". As used herein, a permissive RNA splice signal is an RNA splice signal sequence, preferably a 3' RNA splice signal, from an intron contained within a repertoire of germ line DNA segments which undergo rearrangement during cell differentiation. Examples of such gene repertoires include the immunoglobulin super gene family, including the immunoglobulins and T-cell antigen receptors as well as the repertoire of the major histocompatibility complex (MHC) genes and others. Particularly preferred permissive splice sequences are those obtained from the immunoglobulin repertoire, preferably of the IgG class, and more preferably those 3' splice signal sequences associated with the J-C segment rearrangement of the Ig heavy and light chain, most preferably the heavy chain. A particularly preferred permissive splice sequence comprises that portion of the sequence as shown downstream of the HindIII site in Fig. 11. A particularly preferred hybrid intervening sequence comprises the entire sequence shown in Fig. 11 which includes a 5' portion of an intervening sequence from bovine α S1-casein and a 3' sequence portion of an IgG heavy chain intervening sequence.

Such hybrid intervening sequences containing permissive RNA splice signals are preferably used when the

recombinant DNA corresponds to a cDNA sequence. As indicated in the Examples, when 16kb of 5' expression regulation sequence from the α S1-casein gene was used in conjunction with an α S1-casein-IgG hybrid intervening
5 sequence to express human lactoferrin cDNA operably linked to the α S1-casein secretory signal sequence a transgenic mouse was obtained which produced approximately 1330 μ g/ml of hLF in the transgenic milk. This amount of recombinant polypeptide far exceeds the
10 previously reported amounts for production of various protein in transgenic mouse milk of generally less than 10 μ g/ml and in one case approximately 50 μ g/ml. It also exceeds the maximum of 8 μ g/ml of hLF produced herein when the same transgene was used that contained a
15 homologous bovine intervening sequence rather than the hybrid intervening sequence.

However, such hybrid intervening sequences are not limited to transgenes utilizing cDNA sequence. Rather, hybrid intervening sequences are also useful when the
20 recombinant polypeptide is encoded by a genomic sequence. Based on the results obtained with the cDNA recombinant DNA and the general expectation that genomic DNA sequences express at higher levels than sequences derived from cDNA, it is expected that such hybrid
25 intervening sequences used in conjunction with genomic recombinant DNA will further enhance expression levels above that which would otherwise be obtained with genomic sequence alone.

Based on the foregoing, it is apparent that preferred
30 transgenes include large amounts of 5' and 3' expression regulation sequences. Further, the recombinant DNA is preferably derived from genomic clones which may be tens to hundreds of kilobases in length. Based on the present technology for cloning and manipulating DNA, the
35 construction and microinjection of transgenes is

practically limited to linearized DNA having a length not greater than about 50kb. However, the transgenes of the invention, especially those having a length greater than about 50kb, may be readily generated by introducing two or more overlapping fragments of the desired transgene into an embryonal target cell. When so introduced, the overlapping fragments undergo homologous recombination which results in integration of the fully reconstituted transgene in the genome of the target cell. In general, it is preferred that such overlapping transgene fragments have 100% homology in those regions which overlap. However, lower sequence homology may be tolerated provided efficient homologous recombination occurs. If non-homology does exist between the homologous sequence portions, it is preferred that the non-homology not be spread throughout the homologous sequence portion but rather be located in discrete areas. Although as few as 14 base pairs at 100% homology are sufficient for homologous recombination in mammalian cells (Rubnitz, J. and Subramani, S. (1984) Mol. Cell. Biol. 4, 2253-2258), longer homologous sequence portions are preferred, e.g. 500bp, more preferably 1000bp, next most preferably 2000bp and most preferably greater than 2000bp for each homologous sequence portion.

As indicated in the examples, three overlapping fragments of the human serum albumin gene were microinjected into the pronuclei of mouse zygotes in approximately equal molar portions. These fragments successfully recombined and integrated into the mouse genome as confirmed by analysis of the integrated DNA by Southern blotting procedures and by detection of RNA transcript and human serum albumin in the serum of the transgenic mouse. Although the transgene so generated has a unit length of 38kb, there is no known practical limit to the size of the transgene which may be formed

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using larger and/or greater numbers of overlapping transgene fragments. In particular, it is expected that transgenes may be formed by this approach having lengths between about 50 to 1000kb and more preferably between 5 50 and 500kb. Further, the use of homologous recombination of overlapping fragments is expected to be fruitful in the generation of larger transgenic animals, such as transgenic bovine species, containing transgenes incorporating recombinant DNA comprising 10 genomic DNA which otherwise could not be incorporated into a pronucleus to form a transgenic animal. Such genomic transgenes are expected to produce higher expression levels in transgenic cows as compared to that which is produced by transgenes encoding recombinant 15 cDNA.

When, the ultimate object is to secrete a recombinant polypeptide, a "secretory DNA sequence" encoding a functional secretion signal peptide is also operably linked within the transgene to direct secretion of the 20 recombinant polypeptide from one or more cell types within the transgenic animal. Secretory DNA sequences in general are derived from genes encoding secreted proteins of the same species of the transgenic animal. Such secretory DNA sequences are preferably derived from 25 genes encoding polypeptides secreted from the cell type targeted for tissue-specific expression, e.g. secreted milk proteins for expression in and secretion from mammary secretory cells. Secretory DNA sequences, however, are not limited to such sequences. Secretory 30 DNA sequences from proteins secreted from other cell types within the species of transgenic animal may also be used, e.g., the native signal sequence of a homologous gene encoding a protein secreted other than in the mammary glands. In addition, "heterologous 35 secretory DNA sequences" which encode signal secretion peptides from species other than the transgenic animals

my also be used e.g., human t-PA, human serum albumin human lactoferrin and human lactalbumin and secretion signals from microbial genes encoding secreted polypeptides such as from yeast, filamentous fungi, and
5 bacteria. In general, a secretory DNA sequence may be defined functionally as any DNA sequence which when operably linked to a recombinant DNA sequence encodes a signal peptide which is capable of causing the secretion of the recombinant polypeptide.

10 In one of the preferred embodiments, a secretory DNA sequence encoding a secretory signal sequence functional in the mammary secretory cells of bovine species is used to cause secretion of recombinant polypeptide from bovine mammary secretory cells. The secretory DNA
15 sequence is operably linked to the recombinant DNA sequence. Examples of such secretory DNA sequences include DNA sequences encoding signal secretion sequences for bovine α S1-casein, murine lactoferrin and human transferrin. The preferred secretory DNA sequence
20 is that encoding the secretory sequence of α S1-casein from bovine species. The use of this secretory DNA sequence is described in more detail in the Examples.

"Operably linked" in the context of linking a secretory DNA sequence to a recombinant DNA sequence means that
25 the secretory DNA sequence (comprising codons encoding the secretory signal peptide sequence) is covalently coupled to the recombinant DNA sequence so that the resultant secretory-recombinant DNA sequence encodes 5' to 3' for the secretory signal sequence and recombinant
30 polypeptide. Accordingly, the reading frame for the secretory sequence and the recombinant DNA sequence must be covalently combined such that an open reading frame exists from the 5' end of the mRNA sequence formed after transcription and processing of the primary RNA
35 transcript. This open reading frame in the RNA contains

- a 5' sequence portion encoding the secretory signal peptide and a 3' sequence portion encoding the recombinant polypeptide. When so constructed, the recombinant polypeptide produced upon expression of the secretory-recombinant DNA sequence is of a form which is capable of being secreted from targeted cells which express the DNA sequence. The signal peptide generally is removed in vivo during secretion to produce an extracellular form of the recombinant polypeptide.
- 10 In the preferred embodiments of the invention, a secretory-recombinant DNA sequence is expressed predominantly in the mammary secretory cells of transgenic bovine species. Such tissue-specific expression is obtained by operably linking mammary specific expression regulation DNA sequences to the
- 15 above secretory-recombinant DNA sequence. Such mammary specific regulation sequences include the aforementioned regulation sequences contained in various bovine genes preferentially expressed in the mammary secretory cells
- 20 of the species. Such mammary specific genes include α S1-casein; α S2-casein; β -casein; K-casein; α -lactalbumin; and β -lactoglobulin. Preferred expression regulation sequences are derived from α S1-casein as described more in detail in the Examples.
- 25 In general, the transgenes of the invention that are designed to secrete the recombinant polypeptide into transgenic bovine milk are capable of causing such secretion at levels significantly higher than that previously reported for transgenic mice and sheep. When
- 30 the recombinant polypeptide is encoded by a recombinant DNA corresponding to, or derived from, cDNA, the molar concentration of the recombinant polypeptide is preferably greater than about 1.0 μ M, more preferably greater than about 100 μ M, and most preferably greater
- 35 than 100 μ M. When viewed from the perspective of the

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level of recombinant polypeptide present in the transgenic milk, the amount of recombinant polypeptide is preferably greater than 50 $\mu\text{g/ml}$, more preferably greater than about 500 $\mu\text{g/ml}$ and most preferably greater than about 1000 $\mu\text{g/ml}$ (1mg/ml).

When the transgene of the invention encodes a recombinant polypeptide that is encoded by recombinant DNA derived from or corresponding to genomic DNA (or comprised substantially of such genomic sequences, e.g. greater than about 50%, more preferably greater than about 75%, most preferably greater than 90% of the codons encoding the recombinant polypeptide are from genomic sequences), the molar concentrations and protein levels in bovine transgenic milk are the same as for cDNA or higher. In general, the molar concentration of the recombinant polypeptide in such transgenic milk is preferably greater than about 50 μM , more preferably greater than about 150 μM , most preferably greater than about 500 μM . When viewed from the level of protein in the transgenic milk, the levels are preferably greater than about 10 mg/ml, more preferably greater than about 2.5 mg/ml, most preferably greater than 5 mg/ml.

The foregoing molar concentration and protein levels in bovine transgenic milk will vary depending upon the molecular weight of the particular recombinant polypeptide. A particular advantage of producing a recombinant polypeptide in bovine transgenic milk is that relatively large molecular weight polypeptides may be so produced which are otherwise difficult to produce in large quantities in other systems such as prokaryotic expression systems. Although any recombinant polypeptide may be produced in bovine transgenic milk according to the invention, it is generally preferred that such recombinant polypeptides have a molecular weight greater than about 10,000 Daltons. However,

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other recombinant polypeptides having molecular weights of greater than 15,000, greater than 20,000 and greater than 60,000 Daltons may also be expressed in transgenic bovine milk. For example, human lysozyme having a
5 molecular weight of 17,000 Daltons and lactoferrin having a molecular weight of 79,000 Daltons may be readily produced in the transgenic milk of bovine species according to the disclosure of the invention. Thus, the recombinant polypeptides of the invention have
10 a wide range of molecular weights.

As a consequence, the foregoing preferred molar concentrations of recombinant polypeptides are adjusted when higher molecular weight recombinant polypeptides are produced. Such adjustment is made by converting the
15 molar concentration to the amount of protein produced and adjusting the molar concentrations so that the recombinant protein level is within the following preferred concentrations.

Most of the previous reports relating to the production
20 of polypeptides in transgenic milk involve transgenic mice. The mouse, however, normally produces between 55 to 80 milligrams of protein per ml of milk. A cow, on the other hand, normally produces between 30 to 34 milligrams of protein per ml. Since exceptionally high
25 levels of recombinant polypeptide production may adversely affect the production of endogenous milk protein and/or have adverse effects upon the mammary secretory gland, it is preferred that the recombinant polypeptide concentration be between about 3 and 50% of
30 the normal bovine milk protein concentration (i.e., between about 1 and 17 milligrams of recombinant polypeptide per ml of transgenic milk), more preferably between 10 to 20% (i.e., between 3 to about 7 milligrams per ml) and most preferably between 10 and 15% (i.e.,
35 between about 3 and 5 milligrams per ml) of the normal

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amount of protein produced in bovine milk. Such preferred ranges also provide a preferred maximum limit to the aforementioned levels of protein produced in transgenic bovine milk.

- 5 The above described linking of various DNA sequences to form the transgene of the invention are performed by standard methods known to those skilled in the art or as described herein. Once the transgene or overlapping homologous fragments encoding the transgene are
10 constructed as described they are used to make transgenic non-human animals.

Methods of introducing transgenes or overlapping transgene fragments into embryonal target cells include microinjection of the transgene into the pronuclei of
15 fertilized oocytes or nuclei of ES cells of the non-human animal. Such methods for murine species are well known to those skilled in the art. Alternatively, the transgene may be introduced into an animal by infection of zygotes with a retrovirus containing the transgene
20 (Jaenisch, R. (1976), Proc. Natl. Acad. Sci. USA, 73, 1260-1264). The preferred method is microinjection of the fertilized oocyte. In this preferred embodiment, the fertilized oocytes are first microinjected by standard techniques. They are thereafter cultured in
25 vitro until a "pre-implantation embryo" is obtained. Such pre-implantation embryos preferably contain approximately 16 to 150 cells. The 16 to 32 cell stage of an embryo is commonly referred to as a morula. Those pre-implantation embryos containing more than 32 cells
30 are commonly referred to as blastocysts. They are generally characterized as demonstrating the development of a blastocoel cavity typically at the 64 cell stage. Methods for culturing fertilized oocytes to the pre-implantation stage include those described by Gordon,
35 et al. (1984), Methods in Enzymology, 101, 414; Hogan,

et al. (1986) in Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (for the mouse embryo); and Hammer, et al. (1985), Nature, 315, 680 (for rabbit and porcine embryos)

5 Gandolfi et al. (1987) J. Reprod. Fert. 81, 23-28; Rexroad et al. (1988) J. Anim. Sci. 66, 947-953 (for ovine embryos) and Eyestone, W.H. et al. (1989), J. Reprod. Fert., 85, 715-720; Camous., et al. (1984), J. Reprod. Fert., 72, 779-785; and Heyman, Y., et al.

10 (1987), Theriogenology, 27, 5968 (for bovine embryos). Such pre-implantation embryos are thereafter transferred to an appropriate female by standard methods to permit the birth of a transgenic or chimeric animal depending upon the stage of development when the transgene is

15 introduced. As is well known, mosaic animals can be bred to form true germline transgenic animals.

Since the frequency of transgene incorporation is often low, the detection of transgene integration in the pre-implantation embryo is highly desirable. In one aspect

20 of the invention methods are provided for identifying embryos wherein transgenesis has occurred and which permit implantation of transgenic embryos to form transgenic animals. In this method, one or more cells are removed from the pre-implantation embryo. When

25 equal division is used, the embryo is preferably not cultivated past the morula stage (32 cells). Division of the pre-implantation embryo (reviewed by Williams et al. (1986) Theriogenology 22, 521-531) results in two "hemi-embryos" (hemi-morula or hemi-blastocyst) one of

30 which is capable of subsequent development after implantation into the appropriate female to develop in utero to term. Although equal division of the pre-implantation embryo is preferred, it is to be understood that such an embryo may be unequally divided either

35 intentionally or unintentionally into two hemi-embryos which are not necessarily of equal cell number.

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Essentially, all that is required is that one of the embryos which is not analyzed as hereinafter described be of sufficient cell number to develop to full term in utero. In a specific embodiment, the hemi-embryo which
5 is not analyzed as described herein, if shown to be transgenic, is used to generate a clonal population of transgenic non-human animals.

One of each of the hemi-embryos formed by division of pre-implantation embryos is analyzed to determine if the
10 transgene has been integrated into the genome of the organism. Each of the other hemi-embryos is maintained for subsequent implantation into a recipient female of the species. A preferred method for detecting transgenesis at this early stage in the embryo's
15 development uses these hemi-embryos in connection with a unique property of the restriction endonuclease Dpn I. This enzyme recognizes the sequence GATC in double-stranded DNA but only when the adenine in each strand within this sequence is methylated at N-6. When using
20 this preferred method, the transgene containing the sequence GATC is methylated prior to microinjection either by transferring the transgene on an appropriate plasmid through a DAM⁺ strain of microorganisms such as E. coli MM294 or by directly methylating the transgene
25 with dam methylase. The methylated transgene (preferably without any exogenous sequences such as plasmid vector) is then microinjected into fertilized oocytes (approximately 10 to 500 copies per pronucleus, more preferably 50 to 100 copies per pronucleus). The
30 fertilized oocytes so obtained are cultured in vitro to the pre-implantation stage. During this early growth and cell division phase, the genomic DNA is replicated. Accordingly, those copies of the methylated transgene integrated into the genome of the fertilized oocyte are
35 unmethylated after replication whereas any non-integrated transgenes which may still exist after

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replication will remain methylated. (Lacks, S., et al. (1977), J. Mol. Biol., 114, 153.) This differential methylation pattern for integrated versus non-integrated transgene permits the identification of which fertilized
5 oocytes have integrated the transgene into the genome.

The identification of the pre-implantation embryos containing the integrated transgene is achieved by analyzing the DNA from each of the hemi-embryos. Such DNA is typically obtained by lysing the hemi-embryo and
10 analyzing the thus released DNA after treatment as described by Ninomiya, T. et al. (1989) molecular Reproduction and Development 1, 242-248. Each of the DNA samples is treated with Dpn I. Thereafter, a polymerase chain reaction (Saiki, et al. (1985),
15 Science, 230, 1350-1354) is preformed to amplify all or part of the transgene. When the entire transgene is amplified, two extension primers each complimentary to opposite strands at opposing ends of the transgene are used for amplification. When, however, less than the
20 entire transgene is amplified, such extension primers are chosen such that the amplified gene product spans the Dpn I site in the transgene. If Dpn I cleavage has not occurred, PCR amplification results in amplified sequences having a predetermined size whereas primer
25 extension for those transgenes which have been cleaved will not result in exponential amplification. Generally, the Dpn I/PCR amplified DNA from the hemi-embryo is subjected to electrophoresis followed by hybridization with labeled probe complimentary to the
30 region of the transgene between the two extension primers. This facilitates the determination of the size of the amplified DNA sequences, if any, and provides an indication of whether the transgene has been integrated into the pre-implantation embryo from which the hemi-embryo was obtained (now called a "transgenic hemi-embryo"). If it has, the remaining untreated transgenic
35

hemi-embryo is transplanted into a recipient parent. After in utero development, the transgenic non-human animal having the desired phenotype conferred by the integrated transgene is identified by an appropriate
5 method in utero or after birth. Of course, other restriction endonucleases capable of cleaving a methylated DNA sequence but incapable of cleaving the unmethylated form of a recognition sequence may be used in the aforementioned method.

- 10 The above described method using Dpn I requires that the sequence GATC be present in the transgene of interest. In those cases when such a sequence is not present, it may be readily introduced into the transgene by site directed mutagenesis (Kunkel, T.A. (1985), Proc. Natl.
15 Acad. Sci., 82, 488) or cassette mutagenesis (Wells, J.A., et al. (1985), Gene, 34, 315) provided such mutagenesis does not change the amino acid sequence encoded by the transgene (or causes an inconsequential change in amino acid sequence) and that any codons so
20 generated are functional in the transgenic non-human animal of interest.

The above described methods for the detection of transgenesis in pre-implantation embryos provide economical and time saving method for generating
25 transgenic non-human animals since they significantly decrease the number of pregnancies required to produce a transgenic animal and substantially increase the likelihood that an implanted embryo will produce a transgenic non-human animal. Such methods are
30 especially important for those animals for which very low or non-existent frequencies of transgenesis have been obtained, e.g. bovine species.

In an alternate embodiment, the above described method for detecting transgenesis in pre-implantation embryos

is combined with embryonic cloning steps to generate a clonal population of transgenic embryos which may thereafter be implanted into recipient females to produce a clonal population of transgenic non-human animals also having the same genotype. In this regard, it is to be understood that transgenic embryos and/or non-human transgenic animals having the same "genotype" means that the genomic DNA is substantially identical between the individuals of the embryo and/or transgenic animal population. It is to be understood, however, that during mitosis various somatic mutations may occur which may produce variations in the genotype of one or more cells and/or animals. Thus, a population having the same genotype may demonstrate individual or subpopulation variations.

After a hemi-embryo is identified as a transgenic hemi-embryo, it is cloned. Such embryo cloning may be performed by several different approaches. In one cloning method, the transgenic hemi-embryo is cultured in the same or in a similar media as used to culture individual oocytes to the pre-implantation stage. The "transgenic embryo" so formed (preferably a transgenic morula) is then divided into "transgenic hemi-embryos" which can then be implanted into a recipient female to form a clonal population of two transgenic non-human animals. Alternatively, the two transgenic hemi-embryos obtained may be again cultivated to the pre-implantation stage, divided, and recultivated to the transgenic embryo stage. This procedure is repeated until the desired number of clonal transgenic embryos having the same genotype are obtained. Such transgenic embryos may then be implanted into recipient females to produce a clonal population of transgenic non-human animals.

In a preferred cloning method, the transgenic embryo is cloned by nuclear transfer according to the techniques

- of Prather et al. (1988) Biol. Reprod. 37, 59-86; Roble et al. (1987) J. Anim. Sci. 64, 642-664. According to this method, nuclei of the transgenic embryo are transplanted into enucleated oocytes, each of which is thereafter cultured to the blastocyst stage. At this point, the transgenic embryos may be resubjected to another round of cloning by nuclear transplantation or may be transferred to a recipient parent for production of transgenic offspring having the same genotype.
- 10 In addition to the foregoing methods for detecting early transgenesis, other methods may be used to detect transgenesis. Such methods include in utero and post partum analysis of tissue. In utero analysis is performed by several techniques. In one, transvaginal
- 15 puncture of the amniotic cavity is performed under echoscopic guidance (Bowgso et al. (1975) Bet. Res. 96, 124-127; Rumsey et al. (1974) J. Anim. Sci. 39, 386-391). This involves recovering about 15 to 20 milliliters of amniotic fluid between about day 35 and
- 20 day 100 of gestation. This volume of amniotic fluid contains about 1000 to 12,000 cells per ml originating from the urogenital tract, the skin and possibly the lungs of the developing embryo. Most of these cells are dead. Such cells, however, contain genomic DNA which
- 25 is subjected to PCR analysis for the transgene as an indication of a successful transgenesis. Alternatively, fetal cells may be recovered by chorion puncture. This method also may be performed transvaginally and under echoscopic guidance. In this method, a needle is used
- 30 to puncture the recipient animal's placenta, particularly the placentonal structures, which are fixed against the vaginal wall. Such sampling may be performed around day 60 of gestation in bovine species. Chorion cells, if necessary, are separated from maternal
- 35 tissue and subjected to PCR analysis for the transgene as an indication of successful transgenesis.

- Transgenesis may also be detected after birth. In such cases, transgene integration can be detected by taking an appropriate tissue biopsy such as from the ear or tail of the putative transgenic animal. About one to two centimeters of tail or about five to ten square millimeters of ear are obtained followed by southern blotting with a probe for the transgene according to the method of Hogan et al. (1986) Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory.
- 10 In those embodiments where a recombinant polypeptide is expressed and secreted into the milk of transgenic bovine species, the transgenic milk so obtained may be either used as is or further treated to purify the recombinant polypeptide. This depends, in part, on the
15 recombinant polypeptide contained in the transgenic milk and the ultimate use for that protein. Thus, when the recombinant polypeptide is secreted into transgenic milk to increase the nutritional value of the bovine milk, no further purification is generally necessary. An
20 example of such a situation involves one of the preferred embodiments wherein human lactoferrin is produced in the milk of bovine species as a supplement to control intestinal tract infections in newborn human infants and to improve iron absorption. In other
25 situations, a partial purification may be desired to isolate a particular recombinant polypeptide for its nutritional value. Thus, for example, human lactoferrin produced in transgenic bovine milk may be partially purified by acidifying the milk to about pH 4-5 to
30 precipitate caseins. The soluble fraction (the whey) contains the human lactoferrin which is partially purified.

The recombinant polypeptide contained in bovine transgenic milk may also be used in food formulations.
35 A particularly useful food formulation comprises an

infant formula containing one or more recombinant polypeptides from transgenic bovine milk which have either nutritional or other beneficial value. For example, an infant formula containing human lactoferrin
5 from transgenic bovine milk made according to the present invention provides a bacteriostatic effect which aids in controlling diarrhea in newborn. Similarly, recombinant polypeptides such as human casein and human lysozyme may also be generated in transgenic bovine milk
10 to provide nutritional value. Table 2 sets forth the constituents of a typical infant formula. As indicated therein, the protein content varies between about 1.8 and 4.5 grams of protein per 100 kilocalories of formula. Thus, the total protein including recombinant
15 polypeptide should lie between the values at least based on regulatory requirements in the United States from which the formulation in Table 2 is based. The amount of total protein including recombinant polypeptide, of course, may vary from the foregoing depending upon the
20 local regulations where the particular formula is intended to be used.

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TABLE 2

Nutrient	Minimum ^a	Maximum ^a
Protein (gm) ^f	1.8 ^b	4.5
Fat:		
gm	3.3	6.0
percent cal	30.0	54.0
Essential fatty acids (linoleate):		
percent cal	2.7	
mg	300.0	
Vitamins:		
(A) (IU)	250.0 (75 µg) ^c	750.0 (225 µg) ^c
D (IU)	40.0	100.0
K (µg)	4.0	
E (IU)	0.7 (with 0.7 IU/gm linoleic acid)	
C (ascorbic acid (mg)	8.0	
B ₁ (thiamine (µg)	40.0	
B ₂ (riboflavin) (µg)	60.0	
B ₆ (pyridoxine) (µg)	35.0 (with 15 µg/gm of protein in formula)	
B ₁₂ (µg)	0.15	
Niacin (µg)	250.0	
Folic acid (µg)	4.0	
Pantothenic acid (g)	300.0	
Biotin (µg)	1.5 ^d	
Choline (mg)	7.0 ^d	
Inositol (mg)	4.0 ^d	
Minerals:		
Calcium (mg)	50.0 ^e	
Phosphorus (mg)	25.0 ^e	
Magnesium (mg)	6.0	
Iron (mg)	0.15	
Iodine (µg)	5.0	
Zinc (mg)	0.5	
Copper (µg)	60.0	
Manganese (µg)	5.0	
Sodium (mg)	20.0	60.0
Potassium (mg)	80.0	200.0
Chloride (mg)	55.0	150.0

^aStated per 100 kilocalories.^bThe source of protein shall be at least nutritionally equivalent to casein.^cRetinol equivalents.^dRequired to be included in this amount only in formulas which are not milk-based.^eCalcium to phosphorus ratio must be no less than 1.1 nor more than 2.0.^fIncludes recombinant protein according to the invention or recombinant proteins and other proteins.

In addition to infant formulas, other food formulations may also be supplemented with recombinant polypeptides from transgenic bovine milk. For example, such recombinant polypeptides may be used to supplement
5 common diet formulations.

When the recombinant polypeptide is intended to be used pharmaceutically, purification methods consistent with such an application are called for. Such purification methods will depend on the particular recombinant
10 polypeptide to be purified and are generally known to those skilled in the art. Such methods typically include a partial purification by casein fractionation followed by chromatography of the appropriate fraction containing the recombinant polypeptide. Such
15 chromatography includes affinity chromatography, ion exchange chromatography, gel filtration and HPLC.

In a specific embodiment of the invention, transgenes are provided for producing human lactoferrin in the milk of transgenic bovine species. Human lactoferrin (HLF)
20 is a single chain glycoprotein which binds two ferric ions. Secreted by exocrine glands (Mason et al. (1978) J. Clin. Path. 31, 316-327; Tenovuo et al. (1986) Infect. Immun. 51, 49-53) and polymorphonuclear neutrophil granulocytes (Mason et al. (1969) J. Exp.
25 Med. 130, 643-658), this protein functions as part of a host non-specific defense system by inhibiting the growth of a diverse spectrum of bacteria. HLF exhibits a bacteriostatic effect by chelation of the available iron in the media, making this essential metal
30 inaccessible to the invading microorganisms (Bullen et al. (1972) Br. Med. J. 1, 69-75; Griffiths et al. (1977) Infect. Immun. 15, 396-401; Spik et al. (1978) Immunology 8, 663-671; Stuart et al. (1984) Int. J. Biochem. 16, 1043-1047). This effect is blocked if the
35 protein is saturated with ferric ions. Several studies

suggest that HLF displays a direct bacteriocidal effect on certain microorganisms (Arnold et al. (1980) Infect. Immun. 28, 893-898; Arnold et al. (1977) Science 197, 263-265; Arnold et al. (1981) Infect. Immun. 32, 655-660; Arnold et al. (1982) Infect. Immun. 35, 792-797; Bortner et al. (1986) Infect. Immun. 51, 373-377). The bacteriocidal effect is also inhibited by iron saturation of the protein. No mechanism for the bacteriocidal effect of HLF has been postulated, although it has been demonstrated that it can damage the outer membrane and alter outer membrane permeability in gram-negative bacteria (Ellison et al. (1988) Infect. Immun. 56, 2774-2781).

Lactoferrin is the major iron binding protein in human milk (present at a concentration of about 1.5-1.7 mg/ml) and may play a role in the absorption of iron by the small intestine. All of the iron present in breast milk is thought to be bound to hLF and is taken up at very high efficiencies compared to formula (Hide, D.W., et al. (1981), Arch. Dis. Child., 56, 172). It has been postulated that the high uptake of the hLF bound iron is due to a receptor in the jejunum and data has been presented suggesting existence of receptors in Rhesus monkeys (Cox, et al. (1979), BBA, 588, 120; Davidson, L.A., et al. (1985), Fed. Proc., 18, 901). There is also evidence for specific lactoferrin receptors on mucosal cells of the small intestine of human adults (Cox, et al. (1979) Biochem. Biophys. Acta. 588, 120-128). Free iron levels have been implicated in the control of the intestinal flora (Mevissen-Verhage, et al. (1985), Eur. J. Clin. Microbiol., 4, 14). Breast fed infants, compared with infants fed cow's milk, with and without added iron, were shown to have substantially reduced coliform and, elevated bifidobacteria and clostridia counts in fecal samples. In in vitro studies, human milk has been shown to have a specific

inhibitory effect on E. coli (Brock, et al. (1983), Infect. and Immunit., 40, 453). Human milk has also been shown to have a specific inhibitory effect on E. coli in small intestine due to its high content of iron binding protein, predominantly hLF (Bullen, et al. (1972), British Med. J., i, 69).

Thus, the production of human lactoferrin in the milk of transgenic bovine species provides a source of human lactoferrin. Such lactoferrin may be purified from the transgenic milk for formulation purposes. Alternatively, the whole transgenic milk may be used, preferably after pasteurization, in either liquid or dried form. In addition, the beneficial action of human lactoferrin may be potentiated by combining the human lactoferrin or the transgenic milk containing it with human lysozyme. The human lysozyme may be simultaneously produced in the transgenic cow by introducing a second transgene simultaneously with the HLF transgene to produce a transgenic cow capable of producing more than one recombinant polypeptide in the transgenic milk. Alternatively, the transgenes may be sequentially introduced into bovine species. When such is the case, a transgenic bovine species is obtained containing one of the transgenes. Thereafter, embryonic cells, such as eggs, are obtained from the transgenic female and treated so as to incorporate the second transgene encoding the second polypeptide. Preferably, the egg is fertilized, followed by microinjection of the pronucleus of the zygote so obtained. It is to be understood that the foregoing combination of more than two recombinant polypeptides in transgenic bovine milk is not limited to the aforementioned human lactoferrin and lysozyme combination. Thus, the invention contemplates the production of transgenic bovine species and transgenic milk wherein more than one recombinant

polypeptide is produced by such a transgenic animal in the transgenic milk.

- The complete amino acid sequence of HLF has been determined (Metz-Boutigue et al. (1984) Eur. J. Biochem. 1451, 659-676). HLF comprises two domains, each containing one iron-binding site and one N-linked glycosylation site. These domains show homology between each other, indicative of an ancestral gene duplication and fusion event. In addition, HLF shares extensive homology with other members of the transferrin family (Metz-Boutigue, supra; Pentecost et al. (1987) J. Biol. Chem. 262, 10134-10139). Location of the amino acids involved in the iron-binding sites has been determined by X-ray crystallography (Anderson et al. (1987) Proc. Natl. Acad. Sci. 84, 1769-1773). A partial cDNA sequence for neutrophil HLF was published by Rado et al. (1987) Blood 70, 989-993. There was a >98% agreement between the amino acid sequence deduced from the cDNA and that which was determined by direct analysis of lactoferrin from human milk. The structure of the iron-saturated and iron-free form of human lactoferrin have recently been published. (Anderson, et al., (1989) J. Mol. Biol. 209, 711-734; Anderson, et al. (1990) Nature, 784-787.)
- As used herein, "human lactoferrin" comprises a polypeptide having the amino acid sequence substantially as described by Metz-Boutigue, et al. (1984), Eur. J. Biochem., 1451, 659-676 and as set forth in Fig. 2. It is noted, however, that an earlier partial sequence of the human lactoferrin sequence disclosed a number of discrepancies between the published sequence and that obtained herein. Specifically, the following discrepancies exist (amino acid numbering is from the sequence in Figure 1 with DNA position in parenthesis):

	<u>Amino Acid</u>	<u>Position</u>	<u>In Metz-Boutique</u>
	Arg	122 (418)	Absent
	Thr	130 (442)	Ile
	Gln	151 (505)	Arg
5	Ser	184 (604)	Leu
	Tyr	189 (619)	Lys
	Ser	372 (1169)	Trp
	between Ala and Met	391 (1122)	13 amino acids
10	Cys	403 (1225)	Gly
	Gln	512 (1588)	Glu
	Lys	675 (2077)	Arg

Accordingly, human lactoferrin is also defined by the sequence shown in Figure 1 which combines the sequence differences obtained herein with the published sequence. The term human lactoferrin also includes allelic variations of either of these sequences or recombinant human lactoferrin variants wherein one or more amino acids have been modified by the substitution, insertion or deletion of one or more amino acid residues. In some instances human lactoferrin may be produced in milk with all or part of a secretory signal sequence covalently attached thereto.

As used herein, a "human lactoferrin DNA sequence" is a DNA sequence which encodes human lactoferrin as defined above. Such a human lactoferrin DNA sequence may be obtained from a human mammary gland cDNA library or may be derived from the human genome. Example 2 herein describes the cloning and nucleotide sequence of human lactoferrin derived from a human mammary gland cDNA library. The DNA sequence of this human lactoferrin is shown in Fig. 1 and Fig. 2 and is substantially the same as that described by Rado, et al. (1987), Blood, 70, 989-993. The construction of plasmids containing an expressible transgene encoding hLF is described in the examples. One of these plasmids is cGP1HLF also sometimes referred to as 16,8HLF3)

contains a transgene designed for tissue-specific expression in bovine mammary secretory cells.

In a second embodiment of the invention, transgenes are provided for producing human serum albumin in the milk of transgenic bovine species. Human serum albumin is a serum protein which contains 584 amino acid residues (Minghetti, et al. (1986), J. Biol. Chem., 261, 6747). It is the most abundant protein in human serum and performs two very important physiological functions. Serum albumin is responsible for about 80% of the total osmolarity of blood and it transports fatty acids between adipose tissues.

Human serum albumin is used primarily to expand plasma volume by restoring osmotic pressure in the circulatory system. Currently, a heat treated serum derived hsa fraction is infused in most shock and trauma victims, including most of the patients undergoing extensive surgery. HSA is presently derived from human blood plasma as a by-product from blood fractionation processes to obtain rare blood proteins such as factor VIII and IX. The recently developed technology of producing such factors by biotechnological means, however, threatens the source of human serum albumin.

As used herein "human serum albumin" comprises a polypeptide having the amino acid sequence substantially as that described by Minghetti, et al., ibid; Lawn, et al. (1981), Nucl. Acids Res., 9, 6103. Also included are variations thereof including recombinant human serum albumin variants wherein one or more amino acids have been modified by the substitution, insertion or deletion of one or more amino acid residues. (Minghetti et al. (1986) J. Biol. Chem. 261, 6747-6757.) In some instances, human serum albumin may be produced in milk by expressing a transgene which contains DNA encoding

the secretory signal sequence of hSA. Alternatively, human serum albumin may be produced in and secreted from liver cells of a transgenic animal utilizing a completely heterologous transgene comprising human genomic DNA encoding 5' expression regulation sequences, the human serum albumin secretion signal and structural gene and 3' expression regulation sequences. As indicated in the Examples, transgenes containing this heterologous sequence were formed by in vivo homologous recombination of overlapping transgene fragments to reconstitute the hSA gene in the transgenic animal. The so formed transgenic animal produced human serum albumin in its circulatory system.

As used herein, a "human serum albumin DNA sequence" is a DNA sequence which encodes human serum albumin as defined above. Such a human serum albumin DNA sequence may be obtained from λ HAL-HAI, λ HAL-3W and λ HAL-HI4 as described by Urano et al. (1986) J. Biol. Chem. 261, 3244-3251 and Urano et al. (1984) Gene 32, 255-261 and in the Examples herein.

The human serum albumin DNA sequence was cloned as described in Example 10 herein and subsequently manipulated to substitute for the human lactoferrin gene encoded in plasmid cGP1HLF (also referred to as p16,8HLF4). From this plasmid a transgene is obtained containing 16kb of the 5' expression regulation sequence of the bovine α S1-casein gene, human serum albumin DNA sequence and approximately 8kb of the 3'-flanking region of the α S1-casein bovine gene. This transgene is used to microinject fertilized oocytes from bovine species. After early detection of transgenesis, blastocysts containing the hSA transgene are implanted into a recipient female bovine species and brought to term.

The following is presented by way of example and is not to be construed as any limitation on the scope of the invention.

EXAMPLE 1

5 Construction of a probe specific for bovine α S1-casein sequences.

A. Isolation of Chromosomal DNA

Placental tissue was obtained from the slaughterhouse.
10 Surrounding connective tissue was removed and pieces of about 30 grams were quickly frozen in liquid N₂. Chromosomal DNA was isolated as follows: 30 grams of tissue was homogenized (on ice) with 35 ml of Buffer 1 containing 300 mM Sucrose; 60 mM KCl; 15 mM NaCl; 60 mM
15 Tris.HCl pH 8.2; 0.5 mM spermidine; 0.15 mM spermine; 2 mM EDTA; 0.5 mM EGTA. 65 ml of icecold buffer 1 containing 1% NP40 was added and the mixture was incubated for five minutes on ice. After centrifugation for five minutes at 3000 xg the pellet was rinsed with
20 buffer 1 containing 1% NP40. After repeating the centrifugation step the pellet was resuspended in 5 ml of buffer 1. 5 ml 0.5 M EDTA was quickly added. Final volume was now 15 ml. 0.15 ml of a 10% SDS solution was added. After mixing, RNase A and T1 were added to final
25 concentrations of 0.4 mg/ml and 6 u/ml respectively. After incubation at 37°C for three hours, Proteinase K was added to a final concentration of 0.1 mg/ml. This mixture was incubated for 15 hours at 37°C. The mixture was then carefully extracted with phenol. The aqueous
30 phase was isolated and 1/30 volume of 3M NaOAc pH 5.2 and one volume of isopropylalcohol was added. The precipitate (DNA) was rinsed with 70% ethanol and slowly dissolved in 0.5 ml of 10 mM Tris.HCl pH 8.0; 1 mM EDTA, at 4°C.

B. Amplification of Sequences from the
5'-flanking Region of the α S1-casein Gene

Two DNA-primers were synthesized based on the sequence published by Yu-Lee et al., (1986) Nucl. Acids Res. 14,
5 1883-1902. Primer 1 was located at position-681 relative to the major transcription initiation site and had the following sequence:

5'-TCC ATG GGG GTC ACA AAG AAC TGG AC-3'.
(Seq. ID No.: 5)

10 Primer #2 was located at position +164 relative to the major transcription initiation site and had the following sequence: 5'-TGA AGC TTG CTA ACA GTA TAT CAT
AGG-3' (Seq. ID. No.: 6). The first eight nucleotides
15 contain a HindIII restriction site to facilitate subsequent cloning steps. These primers were annealed to the chromosomal DNA and extended in the presence of deoxynucleotides by TAQ-polymerase. After three minutes the mixture was denatured for one minute at 92°C,
20 reannealed at 50°C for 1.5 minutes and again incubated at extension temperature (68°C) for 2 minutes. This cycle was repeated 30 times. After the last cycle DNA was checked for the presence of the expected EcoRI sites. Both the size of the fragment and the presence
25 of EcoRI sites was as expected. The fragment was then treated with Klenow enzyme to repair any overhanging ends, treated with kinase to attach phosphate groups at the ends of the fragment, incubated at 65°C for 10 minutes to inactivate the kinase and klenow enzymes and
30 finally digested with HindIII. This fragment was then subcloned in pUC19 (Yanisch-Perron, et al. (1985), Gene, 33, 103-109) digested with SmaI and HindIII. Formal proof of the identity of this fragment was obtained by sequencing parts of this subclone (after re-cloning into
35 M13 vector). The determined sequence was identical to

the published sequence. This probe was then used to screen a bovine genomic library to obtain clones specific for the 5'-flanking region of the α S1-casein gene.

5 C. Amplification of Sequences from the 3'-flanking Region of the α S1-casein Gene

A similar approach was taken as described above. Two primers were designed based on the sequence published by Stewart et al (1984) Nucl. Acids Res. 12, 3895-3907.

- 10 The 5'-primer was located just downstream of the coding sequence starting at position 713 of the cDNA sequence. It had the following sequence:

5'-GAG GGA CTC CAC AGT TAT GG-3' (Seq. ID No.: 7).

- 15 The other primer was located at position 1070 of the cDNA sequence and had the following sequence: 5'-GCA CAC AAT TAT TTG ATA TG-3' (Seq. ID No.: 8). These primers were annealed to the chromosomal DNA and the region between these primers was amplified as described above. The resulting fragment was \approx 900 bp longer than
20 expected. Sequence analysis showed that an intervening sequence of this size was present between nucleotide 737 and 738 of the cDNA. The amplified fragment was treated with Klenow-polymerase to repair any overhanging ends and treated with kinase to attach phosphate groups to
25 the ends of the fragment. The fragment was then ligated into pUC19 previously cut with SmaI.

D. Screening of a Bovine Phage Library
for α S1-casein Flanking Sequences

A bovine genomic library, constructed in EMBL3, was obtained from Dr. M. Groenen, Agricultural University
5 Wageningen, Netherlands, and was screened in the following way. The bacteriophage particle titre was determined on Escherichia coli MB406 a permissive host strain (Stratagene Inc.). For this, several dilutions of the phage stock were made in SM buffer (50 mM
10 Tris.HCl pH 7.5, 100 mM NaCl, 10 mM MgSO₄, 0.01% gelatin) and mixed with 200 μ l MB406 (O.D.₅₅₀ = 0.9); after 20 minutes at 37°C, 3 ml top agarose (Luria-Bertani medium, 0.8% agarose, 10 mM MgCl₂) was added and this was plated on LB plates and incubated overnight at
15 37°C.

Approximately 600,000 phages were then plated by adding the required amount of phage stock to 400 μ l MB406. The subsequent plating was as described as above. The next step was transfer of the phage to nitrocellulose
20 filters. Plates were placed at 4°C for one hour. Nitrocellulose filters (S&S) were placed on the top agarose layer and exact position was marked. After lifting, the filters were soaked for (1) 30 minutes in denaturation buffer (1.5M NaCl, 0.5M NaOH); (2) 5
25 minutes in neutralizing buffer (1.5M NaCl, 0.5M Tris.HCl pH 8.0). After rinsing with 2xSSPE (360 mM NaCl, 20 mM NaH₂PO₄, 2 mM EDTA), the filters were baked under vacuum at 80°C for two hours.

Prehybridization of the filters was performed in a
30 buffer containing 50% formamide, 5x Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 5xSSPE, 0.1% SDS and 100 μ g/ml denatured salmon sperm DNA at 42°C for two hours. Hybridization was performed in same buffer at 42°C overnight in a
35 shaking waterbath. The probe, generated as previously

described, was labelled using the Random Primed labelling kit from Boehringer Mannheim. After overnight hybridization the filters were washed three times with 2xSSC, 0.1% SDS at room temperature.

- 5 Overnight exposure of Kodak XAR films was performed with amplifying screens (Dupont) at -70°C. Putative positives were plugged out of the plates and put overnight in SM buffer at 4°C. These were plated out as described above and DNA was isolated following the
- 10 plate lysate method (Maniatis, T., et al. (1982), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y.). 5 ml SM buffer was added to the top agarose layer; after two hours gentle shaking buffer was removed and spun at 4000 rpm at 4°C for 10 minutes.
- 15 Supernatant was transferred to sterile tubes and RNase A and DNaseI (both final concentration 1µg/ml) was added, this was incubated at 37°C for 30 minutes. One volume of a 20% polyethyleneglycol, 2.5 M NaCl solution was added and put on ice for one hour. Centrifugation
- 20 at 4000 rpm for 30 minutes at 4°C left precipitated bacteriophage particles. These were resuspended in 500 ml SM buffer, SDS (final concentration 0.1%) and EDTA (final concentration 5 mM) was added, this was incubated at 68°C for 15 minutes. Protein was removed
- 25 with one phenol and one chloroform extraction step. Precipitation of phage DNA was performed with one volume isopropanol. Phage DNA was washed once with 70% ethanol and dissolved in 50 ml Tris.HCl pH 7.5, 1 mM EDTA buffer.
- 30 Restriction enzyme analysis, agarose gel electrophoresis, transfer of DNA from gel to nitrocellulose filter and Southern blotting were all done according to standard procedures (Maniatis (1982), Molecular Cloning: A Laboratory Manual). Hybridization
- 35 with probes (described hereinafter) was performed

according to the same procedure as the screening conditions described above.

E. Isolation of Clones Containing
5'-flanking Region of Bovine S1-casein

5 Three putative clones were identified using the probe and procedures as described above. After another round of screening, clean recombinant bacteriophage was analyzed. Digestion of cloned DNA with SalI, EcoRI and SalI/EcoRI (double digestion) and hybridization with the
10 probe described above showed identical inserts in all three clones. The insert consisted of an 18kb (partial Sau3A fragment excised with SalI). Transcriptional orientation in the clone was determined with hybridization of above described restriction fragments
15 with (1) probe 1 described above, and (2) the NcoI-NsiI fragment of probe 1. This showed a region of about 16kb upstream of transcription start. Downstream from the transcription start was another 1.9kbp. Sequencing of part of the latter region showed the presence of exon
20 2 and part of intron 2 of the bovine α S1-casein gene. Additional sequencing of the region -103 - +300 confirmed the identity of the clone. The ethidium-bromide pattern of the described restriction fragments also showed the orientation of the clone in the EMBL vector. Subsequent
25 analysis of the clone with the following restriction enzymes (NcoI, PstI, KpnI, BamHI, HindIII, BglII) resulted in the restriction map of 5' flanking region of bovine S1-casein gene as shown in Fig. 3.

F. Isolation of Clones Containing
3'-flanking Region of Bovine α S1-casein

Duplicate nitrocellulose filters from the initial phage plating used for isolating 5' clones were screened with the 3' α S1-casein probe using the same hybridization conditions previously described. Eight positive clones
35 were identified after two rounds of screening. Phage

DNA was prepared as described. Subsequent restriction digests with SaII, EcoRI, and SaI/EcoRI and Southern hybridization with the 3' α S1 probe showed identical inserts in seven of the eight clones. One clone
5 containing an 18.5kb EcoRI insert was further analyzed with the restriction enzymes BsteII and BamHI. A restriction map of that clone is shown in Fig. 4.

EXAMPLE 2

Cloning of Human Lactoferrin Gene

10 A. Materials

Restriction endonucleases, T4 ligase, and T7 polynucleotide kinase were obtained from Boehringer-Mannheim, New England Biolabs, or Bethesda Research Laboratories. Radio-isotopes were purchased from
15 Amersham. A human mammary gland cDNA library in bacteriophage λ gt11 was obtained from Clontech, Inc., Palo Alto, Calif.

B. Isolation of the Human Lactoferrin Gene

The human mammary gland library was screened by standard
20 plaque hybridization technique (Maniatis, et al. (1982) Molecular Cloning: A Laboratory Manual) with three synthetic oligomers. Two of the oligomers were 30-mers corresponding to the cDNA sequence of Rado et al., supra, at amino acid positions 436-445 and 682-691. The
25 third was a 21-mer "best guess" probe based on human codon bias and coding for amino acid sequence of HLF between amino acid residues 18 and 24. Respectively, they were:

(1) 5'-CTTGCTGTGGCGGTGGTTAGGAGATCAGAC-3' (Seq. ID
30 No.: 9)

(2) 5'-CTCCTGGAAGCCTGTGAATTCCTCAGGAAG-3' (Seq. ID No.: 10), and

(3) 5'-ACCAAGTGCTTCCAGTGGCAG-3' (Seq. ID No.: 11).
The probes were radiolabeled (Crouse et al. (1983) Methods Enzymol. 101, 78-98) and used to screen
35

duplicate filters. Filters were washed at a final stringency of 2 X SSC, 37°C.

C. Nucleotide Sequence Analysis

DNA fragments were isolated by use of low-melting
5 agarose (Crouse et al, supra) and subcloned into
bacteriophage M13mp18 or M13mp19 (Messing et al. (1983)
Methods Enzymol. 101, 20-78). The sequence was
determined using the Sequenase enzyme (modified T7 DNA
polymerase) (Tabor et al. (1987) Proc. Natl. Acad. Sci.
10 USA 84, 4767-4771). All reactions were carried out
according to the manufacturer's specifications (US
Biochemicals). The sequence is shown in Fig. 1. The
hLF sequence was digested with HindIII and EcoRI
(present in the surrounding phage sequences) and
15 subcloned into the HindIII and EcoRI site of pUC19 to
form pUS119 Lacto 4.1. This clone contained the entire
coding sequence of the mature form of hLF, but lacked
the complete signal sequence.

EXAMPLE 3

20 Construction of bovine α S1-casein CAT vectors

In order to determine whether the α S1-casein fragments
obtained in Example 1 had promoter and other properties
needed to express a heterologous gene, expression
plasmids were constructed containing variable amounts
25 of 5-' and 3'-flanking regions from the α S1-casein gene.
The chloramphenicol Acetyl transferase gene (CAT) was
used as a heterologous gene in these vector constructs.
The CAT gene is useful to detect the expression level
for a heterologous gene construct since it is not
30 normally present in mammalian cells and confers a
readily detectable enzymatic activity (see Gorman, C.N.,
et al. (1983), Mol. Cell. Biol., 2, 1044-1051) which can
be quantified in the cells or animals containing an
expressible gene.

A. DNA sequences.

681 bp of a α S1-casein promoter plus the first non-coding exon plus approximately 150 bp of the first intervening sequence (IVS) were isolated from a 5'-flanking genomic clone from Example 1 by PCR amplification as an NcoI-HindIII fragment (approximately 830 bp). This fragment is identified as fragment 1 in Fig. 5A. The primer sequences consisted of:

5'-TCCATGGGGGTCACAAAGAACTGGAC-3'
(Seq. ID No.: 12) and
5'-TGAAGCTTGCTAACAGTATATCATAGG-3'
(Seq. ID No.: 13)

that were designed from a sequence published by Yu-Lee et al. (1986) Nuc. Acids Res. 14, 1883-1902.

Approximately 1.6kb (fragment 2, Fig. 5A) of α S1-casein 3'-flanking sequence was isolated by PCR amplification from a bovine 3'-flanking genomic clone from Example 1. This region contained the previously described splice within the 3' untranslated region of α S1-casein gene. Fragment 2 was subcloned into the SmaI site of pUC19. The primer sequences consisted of:

5'-GAGGGACTCCACAGTTATGG-3'
(Seq. ID No.: 14) and
5'-GCACACAATTATTTGATATG-3'
(Seq. ID No.: 15)

that were designed from a sequence published by Stewart et al. (1984) Nucl. Acids Res. 12, 3895-3907.

A hybrid splicing signal comprising the 3' splice site of an immunoglobulin gene (Bothwell et al. (1981), Cell, 24, 625-637) was synthetically prepared and inserted into pUC18 along with unique restriction sites flanking

either side to produce pMH-1. This plasmid is shown in Fig. 6. NcoI and HindIII sites were designed such that ligation with fragment 1 from the bovine 5' genomic clone would result in the functional hybrid splice
5 sequence. See Fig. 11.

A polyadenylation sequence was obtained from SV40 virus as a BamHI-DraI fragment (fragment 3 in Fig. 5A) isolated from pRSVcat (Gorman, C.M., et al. (1982), Proc. Natl. Acad. Sci., 79, 6777-6781).

10 A bacterial CAT coding sequence was subcloned into pUC19 as a PstI-BamHI fragment.

B. Construction of pS13'5'CAT

Fragment 1 of α S1-casein promoter was subcloned into pMH-1 (Fig. 6) between the NcoI and HindIII sites to
15 form pMHS15'flank.

The SV40 polyadenylation sequence (fragment 3) was subcloned as a BamHI-DraI fragment into pUC19 immediately 3' to the 3' α S1-casein flanking sequence (fragment 2) to form pUC19 3' UTR/SV40. This allowed
20 for the removal of a continuous EcoRI-SalI fragment (containing the 3'-flanking sequence and poly (A) sequence) that was subcloned into pMH-1 to derive pMHS13'UTR (Fig. 5B) which was used later to construct pMHSI 3' UTR hlf which contains sequences encoding human
25 lactoferrin.

The EcoRI-SalI sequence (fragments 2 and 3) were subcloned into the EcoRI-SalI sites of pMHS15'flank to form pS13'5'flank.

The PstI-BamHI CAT fragment (fragment 4 in Fig. 5B),
30 after blunting the BamHI site with Klenow, was subcloned

into pS13'5'flank (Fig. 5B) between the PstI and SmaI sites to form pS13'5'CAT.

C. Construction of pS15'CAT

The CAT fragment (fragment 4 in Fig. 5B, PstI-BamHI) and SV40 polyadenylation fragment (fragment 3 in Fig. 5A, BamHI-DraI) were subcloned into the PstI and SmaI sites of pMHS15'flank to form pS15'CAT (Fig. 5C).

D. Assay for CAT Production

Each of these CAT plasmids were transfected into human 293S cells (Graham, F.L., et al. (1977), J. Gen. Virol., 36, 59-72) by the calcium phosphate co-precipitation method (Gorman, C.M., et al. (1983), Science, 221, 551; Graham, F.L., et al. (1973), Virology, 52, 456-467). Cells were harvested 44 hours after transfection and cell extracts were assayed for CAT activity (Gorman, C.M., et al. (1982), Mol. Cell. Biol., 2, 1011; deCrombrughe, B., et al. (1973), Nature [London], 241, 237-251, as modified by Nordeen, S.K., et al. (1987), DNA, 6, 173-178). A control plasmid expressing CAT driven by the Cytomegalovirus Immediate early promoter (Boshart, M., et al. (1985), Cell, 41, 521) was transfected into human 293 S cells to assay for transfected efficiency.

pS13'5'CAT was expressed in these cells at a level which was approximately 30-100 fold lower than the control plasmid, but significantly higher than background. Primer extension analysis indicated that transcription had initiated predominantly in the expected region.

When pS15'CAT was transfected into 293S cells, expression was also detected.

EXAMPLE 4Bovine α S1-casein/human lactoferrin
expression cosmid CGP1HLFA. Construction of DNA Sequences.

- 5 16kb of bovine α S1-casein 5'-flanking sequence from Example 1 was isolated from the bovine genomic library (phage GP1) as a SalI-BglII fragment. The BglII site lies at the junction of the first intron and second exon of the α S1-casein gene.
- 10 Bovine α S1-casein signal sequence (Stewart et al. (1984) Nucl. Acids Res. 12, 3895) was prepared from synthetic DNA synthesized on a Cylone Plus® DNA Synthesizer (Millgen/Biosearch I) and contained the entire signal sequence plus XhoI and Cla I sites attached to the
- 15 5'-end, and NaeI to the 3'-end (fragment 8, Fig. 7B).

- Cleavage of pUC119 Lacto 4.1 with EaeI precisely opened the plasmid at the codon for the first amino acid of mature hLF. Treatment with Klenow was used to fill in the overhanging 5'-end. Further digestion with AccI and
- 20 EcoRI gave two fragments: (a) an EaeI-AccI fragment containing the first 243 bp of mature hLF (fragment 5, Fig. 7C), and (b) a contiguous AccI-EcoRI fragment (fragment 6, Fig. 7C) of 1815 bp that contained all but five terminal codons of the remaining coding sequence.

- 25 A synthetic linker was prepared that contained the last five codons of hLF beginning at the EcoRI site and extending for four bases beyond the stop codon. A KpnI site was added to the 3'-end (fragment 7 in Fig. 7C).

- An 8.5kb EcoRI 3'-fragment was isolated from the bovine
- 30 genomic library (Fig. 4) containing sequences beginning just downstream of the coding region of α S1-casein and a BstEII site approximately 350 bp from the 5'-end. This fragment was subcloned into pMH-1 at the EcoRI site

to form pMH3'E10 (Fig. 7A). A SalI site is adjacent to the 3'-EcoRI site in pMH3'E10.

B. Construction of cGP1HLF

The hLF 3'-linker (fragment 7, Fig. 7C) was subcloned
5 into the EcoRI-KpnI sites of pMH3'UTR (Fig. 7A) to
produce pMH3'UTRhLF2linker (Fig. 7A).

The synthetic bovine α S1-casein signal sequence
(fragment 8) was then subcloned into the XhoI and SmaI
sites of pMH3'UTRhLF2linker to make pS13'hLF1/2L (Fig.
10 7B).

The two hLF coding fragments (fragments 5 and 6 in Fig.
7C) were subcloned into the NaeI and EcoRI sites of
pS13'hLF1/2L (Fig. 7B) to make pS13'UTRhLF (Fig. 7C).

The large α S1-casein 3'UTR fragment from pMH3'E10 (Fig.
15 7A) was isolated as a BstEII-SalI fragment and subcloned
into the same sites of pS13'UTRhLF to form pHLF3'10kb
(Fig. 7D).

Cosmid cGP1HLF was prepared from a 3-way ligation (Fig.
7F):

20 (1) the 16kb 5'-flanking sequence from phage
GP1 (Example 1, Fig. 3) was modified by attaching two
linker adapters. The SalI site at the 5'-end was
ligated to a NotI-SalI linker. The BglII site at the
3'-end was ligated to a BglII-XhoI linker;

25 (2) the hLF coding region, flanked on the 5'-end
by the α S1-casein signal sequence and on the 3'-end
by approximately 8.5kb of α S1-casein 3'-flanking
sequence, was isolated as a XhoI-SalI fragment from
pHLF3'10kb. The SalI site at the 5'-end was ligated
30 to a SalI-NotI linker;

(c) Cosmid pWE15 (Stratagene, Inc.) was linearized with NotI.

Fragments from (a), (b), and (c) were ligated together and transfected into bacteria using commercial lambda packaging extracts (Stratagene, Inc.) to produce CGP1HLF.

EXAMPLE 5

Bovine α S1-casein/hLF expression plasmids.

A. Construction of pS13'5'hLF

10 The HindIII-SalI fragment of pS13'UTRhLF was subcloned into the same sites in pMHS15'flank to form pS13'5'hLF (Fig. 7E). This plasmid contains 681 bp of bovine α S1-casein promoter sequence, the α S1-casein/IgG hybrid intron, the α S1-casein signal sequence, the hLF coding region, approximately 1.6kb of α S1-casein 3'-flanking sequence, and the SV40 late region polyadenylation sequence.

B. pS15'hLF

Plasmid pS13'5'hLF (Fig. 7E) was cut with KpnI and BamHI which border the α S1-casein 1.6kb 3'-flanking sequence. The larger vector fragment was purified, made blunt ended with Klenow, and self-ligated to form pS15'hLF.

C. Radioimmunoassay for hLF

25 An immunoglobulin-enriched fraction of ascites fluid of a monoclonal antibody against human lactoferrin, which does not cross-react with the bovine or murine protein, was prepared by 50% ammonium sulfate precipitation and coupled to CNBr-activated Sepharose 4B (20 mg of protein to 1 g of Sepharose). The Sepharose beads were 30 suspended (2 mg/ml) in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 0.14 M NaCl containing 10 mM EDTA, 0.1% (v/v) Polylorone and 0.02% (v/v) NaN₃, pH 7.4. Sepharose suspensions (0.3 ml) were incubated for five

hours at room temperature by head-over-head rotation with samples (usually 50 μ l) in 2-ml polystyrene tubes. Sepharose beads were then washed with saline (five times with 1.5 ml) and incubated for 16 hours at room temperature with 50 μ l (1kBq) of 125 I-labeled-affinity-purified polyclonal rabbit anti human lactoferrin antibodies, together with 0.5 ml of PBS, 0.1% (v/v) Tween-20. Thereafter the Sepharose was washed again with saline (four times with 1.5 ml) and bound radioactivity was measured. Results were expressed as percent binding of the labelled antibodies added. Levels of lactoferrin in test samples were expressed in nanomolar, using purified human milk lactoferrin as a standard (serial dilutions in PBS, 10 mM EDTA, 0.1% (v/v) Tween-20.

Repeated testing of standard on separate occasions revealed that this RIA was highly reproducible, intra- and inter assay coefficients of variation ranged from 5-10%. As little as 0.1 nanogram human lactoferrin is easily detected by this RIA.

D. Expression in 293S cells

293S cells were transfected with the above hLF plasmids as described (1 μ g of a CMV-CAT plasmid was co-transfected as control for transfection efficiency). Forty-four hours after transfection medium was removed from the cells and assayed for hLF as described supra, RNA was isolated as described by Stryker, et al. (1989) EMBO J. 8, 2669. The results can be summarized as follows:

1. Transfection efficiencies are identical for the two hLF plasmids;
2. hLF is expressed in the cells and secreted into the medium. In both cases, the levels are about 0.4 μ g/ml medium using about 3×10^6 cells

3. The proteins behave identical to hLF in a human milk sample in a dose response assay measuring the amount of ^{125}I - anti-lactoferrin bound as a function of the amount of sample used.

5 4. The protein has about the same size (~80kD) as in a human milk sample as judged by Western blotting.

5. The hLF RNA produced in the cells has the correct size and its level is similar for both plasmids as judged by Northern - blotting.

10 These data indicate that these two expression plasmids are able to express hLF. By all standards used so far, the protein is identical to hLF present in human milk. The heterologous signal sequence is functional in that
15 it promotes secretion of the protein from the cells into the medium. Further, the casein regulatory sequences used in these plasmids are able to promote expression of a heterologous gene.

EXAMPLE 6

In vitro Maturation, Fertilization 20 and Culture of Bovine Oocytes

Immature oocytes are obtained in large quantity (400-600/day) by aspirating follicles of ovaries obtained at abbatoirs. Immature oocytes are cultured
25 for a period in vitro before they are competent to be fertilized. Once "matured", oocytes are fertilized with sperm which has also been matured, or "capacitated" in vitro. The pronuclei of the fertilized oocyte is then injected with the transgene encoding for the expression
30 and secretion of human lactoferrin. Zygotes resulting from this in vitro fertilization and microinjection are then cultured to the late morula or blastocyst stage (5-6 days) in medium prepared, or "conditioned" by oviductal tissue. Blastocysts are then transferred non-
35 surgically to recipient cattle for the balance of

gestation or analyzed for integration of the transgene as described herein.

In vitro maturation (IVM). Ovaries are obtained immediately after slaughter at local abbatoirs and
5 oocytes are recovered. Alternatively, oocytes are obtained from living cattle by surgical, endoscopic, or transvaginal ultrasonic approaches. In all cases, oocytes are aspirated from ovarian follicles (2-10 mm diameter). After washing, oocytes are placed in a
10 maturation medium consisting of M199 supplemented with 10% fetal calf serum, and incubated for 24 hours at 39°C. Sirard et al. (1988) Biol. Reprod. 39, 546-552.

In vitro fertilization (IVF). Matured oocytes are fertilized with either fresh or thawed sperm. Sperm are
15 prepared for fertilization by first obtaining a population of sperm enriched for motility by a "swim-up" separation technique (Parrish et al. (1986) Theriogenology 25, 591-600). Motil sperm are then added to a fertilization media, consisting of a modified
20 Tyrode's solution (Parrish et al. (1986) supra.) supplemented with heparin to induce sperm capacitation (Parrish et al. (1988) Biol. Reprod. 38, 1171-1180). Capacitation constitutes the final sperm maturation process which is essential for fertilization. Sperm and
25 oocytes are co-cultured for 18 hours. A useful feature of this IVF method is that (in the case of frozen sperm) consistent, repeatable results are obtained once optimal fertilization conditions for a particular ejaculate have been defined (Parrish et al. (1986) supra.).

30 In vitro culture (IVC). Conventional culture systems, which support development of murine, rabbit, or human ova, do not support development of bovine embryos past the 8-16 cell stage. This problem has been overcome by pre-conditioning culture media with oviductal tissue.

Oviduct-conditioned medium will support bovine embryos past the 8-16 cell stage to the blastocyst stage in vitro (Eyestone and First (1989) J. Reprod. Fert. 85, 715-720).

- 5 Bovine embryos have proved refractory to in vitro culture. This in part stems from the existence of a "block" to cleavage in vitro at the 8-16 cell stage. This block may be alleviated by culturing embryos in the oviducts of rabbits (reviewed by Boland (1984)
- 10 Theriogenology 21, 126-137) or sheep (Willadeen (1982) in: Mammalian Egg Transfer, (E. Adams, ed., pp. 185-210)); Eyestone et al. (1987) Theriogenology 28, 1-7). However, these in vivo alternatives have been less than ideal, in that: (1) they require the
- 15 maintenance of large numbers of recipient animals, (2) they require surgery to gain access to the oviducts for transfer, and a second surgery (or sacrifice) to recover the embryos, (3) all transferred embryos are seldom recovered, and (4) access to embryos during
- 20 culture for observation or treatment is entirely precluded. The lack of in vitro culture systems has hampered the development of various manipulation techniques (such as gene transfer by pronuclear injection) by preventing accumulation of basic
- 25 information of the chronology and ontogeny of bovine development, and by complicating the process of culturing embryos to a stage compatible with non-surgical embryo transfer and cryopreservation techniques (e.g., late blastocyst stages).
- 30 Bovine embryos did not yield to attempts to culture them in vitro past the 8-16 cell "block" until Camous et al. (1984) J. Reprod. Fert. 72, 479-485 demonstrated cleavage to 216 cells when embryos were co-cultured with trophoblastic tissue.

The co-culture procedure was extended to oviductal tissue, based on the ability of homo- or hetero-oviducts to support development from zygote to blastocyst. Thus, bovine embryos co-cultured with oviductal tissue, or in
5 medium conditioned by oviductal tissue, developed from zygote to blastocyst in vitro (Eyestone and First, (1989) J. Reprod. Fert. 85, 715-720; Eyestone W.H. (1989) "Factors affecting the development of early bovine embryos in vivo and in vitro." Ph.D. Thesis,
10 University of Wisconsin). Blastocysts have been produced in this system after superovulation and artificial insemination, or by in vitro maturation (IVM), and fertilization (IVF) of immature oocytes. Blastocysts produced in this fashion resulted in
15 pregnancies and live calves after transfer to recipient animals. The results obtained were as follows:

<u>Step</u>	<u>Efficiency</u> <u>(%)</u>	<u>Number</u> <u>(per 100)</u>
IVM	90	90
20 IVF	80	72
IVC	30	22
Embryo transfer (% pregnant)	50	11

Therefore, from an initial daily harvest of 500 oocytes,
25 it is expected the approximately 55 pregnancies will result.

Preparation of Oviduct Tissue
Co-Culture and Conditioned Medium

1. Obtain bovine oviducts after slaughter or by
30 salpingectomy.
2. Harvest luminal tissue by scraping intact oviduct gently with a glass slide.

3. Wash tissue 5 times in 10 ml modified tyrodes-hepes solution (Parrish et al. (1988) Biol. Reprod. 38, 1171-1180).
4. Resuspend final tissue pellet in M199 + 10% fetal calf serum at a ratio of 1 volume tissue:50 volumes of media.
5. Tissue suspension can be used for embryo-co-culture.
6. Alternatively, media may be conditioned for 48h; after centrifuging the suspension, the supernatant may be used as embryo culture medium. Conditioned medium may be stored at -70°C, if desired. Conditioned medium should be used at full strength for embryo culture (no dilution) (Eyestone (1989) ibid).

15

EXAMPLE 7Microinjection of hLF Transgene into Bovine Pronuclei

- The DNA fragment containing the hLF expression unit is excised from the vector by digestion with the appropriate restriction enzyme(s) and separated on agarose gels. The fragment is purified by electroelution, phenol and chloroform extraction and ethanol precipitation (Maniatis et al.). The DNA fragment is dissolved in and dialyzed in 10 mM tris, 0.1 mM EDTA pH 7.2 at a concentration of 1 to 2µg/ml.
- Microinjection needles are filled with the dialyzed DNA solution.

- Before in vitro fertilization, cumulus cells are removed from the egg by either vortexing at maximal speed for 2 minutes or pipetting the eggs up and down several times in a standard micropipet. Bovine pronuclei are injected in principle as murine pronuclei (Hogan, B. et al. (1986) in: Manipulating the mouse embryo, Cold Spring Harbor Laboratory) with an additional centrifugation step in order to visualize the pronuclei.

-65-

The injection takes place 18-24 hours after fertilization. The time varies depending on the bull used as a source of semen. Different batches of semen cause the nuclei to become visible at different times.

- 5 Bovine oocytes, matured and fertilized in vitro, are spun in an eppendorf tube in 1 ml of tyrodes-hepes solution (Parrish (1987)) at 14500 g for eight minutes (Wall et al. (1985) Biol. Reprod. 32, 645-651). The embryos are transferred to a drop of tyrodes-hepes
10 solution on a microscope slide covered with paraffin oil. Using a hydraulic system the oocytes are fixed to the egg holder in such a way that both the pronuclei are visible (using interference-contrast or phase contrast optics). If necessary, the oocytes are rolled to change
15 their position on the egg holder to visualize the pronuclei. The injection needle is brought into the same sharp focus of one of the pronuclei. The needle is then advanced through the zona pellucida, cytoplasm into the pronucleus. A small volume of 1-3 pl is
20 injected (containing 20-100 DNA copies) into the pronucleus either by using a constant flow or a pulse flow (using a switch) of DNA solution out of the needle. Alternatively, two cell stage embryos are spun as described and the nuclei of both blastomers are injected
25 as described. The injected embryos are then transferred to a drop of co-culture medium as described in Example 6 in order to develop to the morula or blastocyst stage.

EXAMPLE 8

Early Detection of Transgenesis with hLF Transgene

- 30 Upon the microinjection of a construct, the oocyte is cultured. A proper site of each embryo is cleaved and subjected to lysis (King, D. et al. (1988) Molecular Reproduction and Development 1, 57-62), proteolysis (Higuchi, R., (1989) "Amplifications (A forum for PCR
35 Users." 2, 1-3) and DPNI digestion. PCR is performed

as described previously (Ninomiya, T. et al. (1979) Molecular Reprod. and Devel. 1, 242-248) with sets of two primers, one in α S1 and the other in hLF cDNA sequence. For example, in a PCR where the forward primer (30mer) α S1 sequence is

ATG AAA CTT ATC CTC ACC TGT CTT GTG

(Seq. ID No.: 16)

and the reverse primer (30mer) in hLF sequence is GGG TTT TCG AGG GTG CCC CCG AGG ATG GAT (Seq. ID No.: 17); 971-1000 of Figure 1), a 990 bp fragment will be generated. This fragment contains the hitherto inactivated DpnI site by loss of adenosine-methylation, at 934 bp away from the start of the forward primer.

EXAMPLE 9

Production of hLF in Milk of Bovine Species

Bovine morula developed from microinjected oocytes are split according to the method of Donahue (Donahue, S. (1986) Genetic Engineering of Animals, ed. J. Warren Evans et al., Plenum). One half of the morula is kept in culture to develop into blastocysts. The other half is subjected to the DNA analysis as described in Example 8. When the result of this analysis is known, the morula kept in culture are developed into a blastocyst or as a source for nuclear transfer into enucleated zygotes. Blastocyst transfer into synchronized cows is performed according to the method of Betteridge (Betteridge, K.J. (1977) in: Embryo transfer in farm animals: a review of techniques and applications).

hLF is detected in the milk of lactating transgenic offspring using the RIA of Example 5.

EXAMPLE 10Bovine α S1-casein/hSA Expression Plasmids

Three overlapping phage clones that contain the complete hSA gene are used to construct an expression vector for hSA. They are designated λ HAL-HA1, λ HAL-3W and λ HAL-H14. They are described in Urano, et al. (1986), J. Biol. Chem., 261, 3244-3251; and Urano, et al. (1984), Gene, 32, 255-261. The sequence of the gene plus some surrounding regions is published in Minghetti, et al. (1986), J. Biol. Chem., 261, 6747-6757. A single phage containing the complete hSA gene is constructed as follows:

Clone HA-1 is cut with BstEII and AhaII. The \approx 1400 bp fragment running from position 1784 (in the first exon, just downstream of the ATG) to 3181 is isolated and a synthetic linker is attached to the BstEII site at the 5' end containing the first few amino acids that are cut off with BstEII as well as the sequence surrounding the ATG as well as a few convenient restriction sites. This fragment is called fragment #1.

Clone 3W is cut with AhaII and SacI the \approx 13.1kb fragment running from position 3181 to 16322 is isolated and a synthetic linker is attached to the SacI site to facilitate cloning in phage EMBL3. This fragment is called fragment #2.

These two fragments are ligated and cloned in phage EMBL3. After identification of the correct phage, a fragment running from just upstream of the BstEII site (where unique restriction sites have been introduced) to the SacI site are isolated and ligated from a SacI to SalI fragment (running from position 16322 to \approx 21200 isolated from clone H-14. These two fragments are then ligated and cloned in EMBL4.

After cutting with ClaI (just upstream of the BstEII site, newly introduced) and BamHI (just downstream of the SaII site in the phage DNA) this new clone yields a fragment containing the complete hSA gene with about
5 2.5kb 3'-flanking sequence.

To construct an expression vector for hSA cosmid cGP1HLF is partially digested with ClaI and BamHI. This removes the signal sequence, the coding sequence of hLF, the 3'-UTR and poly(A) addition region of α S1-casein as well
10 as a small region 3' of the casein gene.

This is ligated to the hSA fragment described above and the resulting cosmid is called cGP1HSA.

The expression vector so formed contains, (1) 16kb of promoter sequences derived from the α S1-casein gene,
15 (2) the first exon and intervening sequence of this gene both present in GP1, (3) the signal sequence of the hSA gene the complete genomic gene coding for hSA including 2.5kb downstream of that gene, and (4) \approx 8kb of 3'-flanking sequence derived from the α S1-casein gene.

20 This transgene is used to produce transgenic bovine species producing hSA in their milk in a manner analogous to that used to produce hLF in the milk of bovine species.

EXAMPLE 11

25 Purification of HSA from the Milk of Bovine Species
Purification of heterologous proteins from milk is facilitated by the fact that, following casein precipitation, those proteins, for the most part, are found in the whey fraction which is less contaminated
30 than the production media used in microbial or cell-based systems.

Chromatographic techniques are preferred for the purification of hSA from cow milk. This approach produces a better recovery and higher albumin purity as well as a lower content of albumin polymers as compared with ethanol fractionation (Curling (1980) in: "Methods of Plasma Protein Fractionation", Curling, ed., Academic Press London, UK; Curling et al. (1982) J. Parenteral Sci. Technol. 36, 59; Berglof et al. and Martinache et al. (1982) Joint Meeting IHS-ISBT, Budapest). The specific transport role of hSA as well as its major role in maintaining intravascular osmotic pressure may also be better preserved upon chromatographic purification (Steinbruch (1982), Joint Meeting ISH-ISBT, Budapest).

The following steps are used to recover hSA produced in the milk of transgenic cows:

1. Precipitation of caseins (about 80% of milk protein) and essentially all the milk fat at pH 4.5 and/or by adding chymosin. The whey fraction contains the albumin;
2. Affinity-chromatography of albumin on Cibacron blue 3GA-Sepharose CL-6B (Harvey (1980) in: Methods of Plasma Protein Fractionation, op. cit.) This step serves both to remove proteins other than albumin and to decrease the volume to be handled about 30-fold. Albumin is eluted from this matrix with 0.15 M NaCl and 20 mM sodium salicylate at pH 7.5;
3. Buffer-exchange on Sephadex G-25: desalting into 0.025 M sodium acetate, adjustment to pH 5.2, followed by filtration;
4. Anion-exchange chromatography on DEAE-Sepharose CL-6B. Desorption of albumin at pH 4.5;
5. Cation-exchange chromatography on CM-Sepharose CL-6B. Albumin elution with 0.11 M sodium acetate,

pH 5.5 and concentration of albumin at a 6% (w/v) solution by ultrafiltration; and

6. Gel filtration on Sephacryl S-200. Fraction of high-molecular weight protein (e.g. albumin polymers, pyrogens) is discarded. The main fraction (albumin monomers) is concentrated by ultrafiltration and formulated.

It is to be noted that steps 3-6 are essentially identical to the method described by Curling and others (Curling (1980) op. cit.; Curling et al. (1982) op. cit.; Berglöf et al. (1982) op. cit.) for the purification of hSA from plasma.

EXAMPLE 12

Transgenic Mice Containing the Human Serum Albumin (hSA) Transgene Generated by Homologous Recombination

Three overlapping genomic hSA clones were used to generate the hSA gene in transgenic mice, λ HAL-HA1, λ HAL-H14 and λ HAL-3W, are shown in Figure 8 as reported by Urano, et al. (1984), Gene, 32, 255-261 and Urano, et al. (1986), J. Biol. Chem., 261 3244-3251. Briefly, a genomic library was constructed from a partial EcoRI digest of human fibroblast DNA. For the clones λ HAL-H14 and λ HAL-3W, this library was screened with 32 P-labeled human albumin genomic clones by hybridization in 1 M NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.1% SDS, 100 ug/ml of sheared salmon sperm DNA and 10x Denhardt's solution at 65° C overnight after prehybridization in 3x SSC and 10x Denhardt's solution. Following hybridization, filters were washed in 0.2x SSC and 0.1% SDS at 65° C. The isolation of the λ HAL-HA1 clone was identical except that a 0.9 kb BglII-EcoRI fragment from the 5' end of λ HAL-3W was used to screen the human fibroblast library.

These three hSA phage clones were used to generate three overlapping linear DNA fragments, which in composite comprised the whole HSA gene and flanking regions. The 5' most fragment I was a EcoRI-EcoRI fragment isolated from λ HAL-HA1; the middle fragment II was a AcyI (=AhaII) -SacI fragment of λ HAL-3W; and the 3' most fragment III was a XhoI-SalI fragment of λ HAL-H14 (Fig. 7). The fragments were treated with klenow DNA polymerase and dNTP's to fill in overhanging sticky ends. In some experiments, the blunt ended fragments were then treated with bacterial alkaline phosphatase to remove the 5' phosphate groups from each fragment. The overlapping DNA fragments were next concentrated then coinjected into the male pronuclei of fertilized mouse eggs, according to published methods (Hogan, et al. (1986) in "Manipulating the Mouse Embryo: A Laboratory Manual", Cold Spring Harbor Laboratory). While the number of molecules injected varied from ≈ 25 to ≈ 100 per egg cell, the ratio of the individual fragments was approximately 1:1:1. Embryos were implanted into the uteri of pseudo pregnant female mice according to the methods of Hogan, et al., supra.

To assay correct homologous recombination of the three overlapping fragments and integration of the nascent transgene into the mouse genome, genomic DNA from the newborn pups was subject to the following specific digestions followed by Southern hybridization with HSA cDNA probes:

Bst EII: cuts outside the HSA gene region and yields an 18 kb band if correct recombination occurred;

Nco I: cuts outside the overlapping regions and yields bands of 8.0 and 9.3kb if correct recombination occurred;

Nco I + Hind III: cuts at several positions outside the region of overlap, indicative of the presence of intact fragments;

5 Hinc II: cuts in the overlapping regions, yielding several bands indicative of correct arrangement in these regions.

10 In an initial experiment of 28 transgenic animals born, 22 had correctly recombined all three fragments. From 20 out of those 22 animals blood was collected and
15 assayed for the presence of hSA protein using a radio immuno assay. 15 out of those 20 animals showed hSA expression at levels between 0.5 and 5 μ g/mL. None of the animals that had no recombination or that were not transgenic showed any expression. Using RNA blots, only
15 two (the two with the highest protein level) showed a band. We are currently performing blots on RNA that has been enriched for the presence of mRNA (i.e., poly(A)+ RNA). Using reverse transcriptase to synthesize cDNA, followed by PCR, we have observed a perfect relationship
20 between the presence of RNA and protein. However, in this experiment we could not determine the size(s) of the RNA.

EXAMPLE 13

Alternate Construction of Transgenes Encoding hLF

25 This example describes the construction of two hLF transgenes wherein the first contains approximately 16kb of α S1-casein 5' expression regulation sequence (pGP1hLF (16kb) also referred to as p16,8HLF4) and the second contains approximately 7.9kb of α S1-casein 5' expression
30 regulation sequence (pGP1hLF (8kb) also referred to as p8.8HLF4). The overall strategy for these constructions is depicted in Fig. 9.

- A 1.8kb EcoRI-BglII fragment (fragment C in Fig. 9) was isolated from phage clone GP1. This fragment runs from position -100 of the transcription start site into the second exon of the α S1-casein gene. The BglII site lies at the junction of the first intron and second exon of the α S1-casein gene. The 3' end containing the BglII site was ligated to a synthetic BglII-ClaI linker and subcloned into the plasmid pUC19. The resulting plasmid is designated pEBS.
- 10 Fragment B in Fig. 9 was isolated as an EcoRI fragment and cloned into the EcoRI site of pEBS. Fragment B includes sequences from position -7500 to position -100 of the transcription start site in the α S1-casein gene. The plasmid so formed is designated pEB3S and contains the combination of fragments B and C is the 8.9kb EcoRI-ClaI fragment running from position -7500 to position +1400 of the transcription start site. The 8.9kb EcoRI-ClaI fragment from pEB3, obtained by complete digestion with ClaI and partial digestion with EcoRI was isolated and subcloned into EcoRI-ClaI cut pKUN2 (a derivative of pKUN; Gene (1986) 46, 269-276 containing a NotI restriction site) to form pNE3BS.

25 An 8.5kb ClaI-EcoRI fragment (fragment A in Fig. 9) running from position -16000 to position -7500 of the transcription start site was isolated from phage GP1. It was thereafter subcloned into pUC19 to form pSE. Using synthetic oligonucleotide, a unique NotI site was introduced into the ClaI site thereby destroying it. The resulting plasmid is designated pNE.

- 30 The insert from pNE was isolated as a NotI-EcoRI fragment and together with the EcoRI-ClaI insert from pNE3BS was ligated into the cloning vector pKUN2. The resulting plasmid pGP1 (Δ 2ex) contains 16kb of α S1-

casein promoter plus the 5' end of the gene to the BglII site at the border of the second exon.

The final plasmid (16,8HLF4) containing the transgene was assembled using the NotI-ClaI fragment from clone pGPI ($\Delta 2ex$) and the Xho-NotI fragment from clone pHLF 3' 10kb. The structure of this transgene is the same as previously described herein.

As a minor modification to this plasmid the SalI site of this plasmid was removed by cutting with SalI and inserting a linker that contains a NotI site, but not a SalI site. Subsequently, a SalI site was introduced just downstream of the hLF sequence by cutting the KpnI site at that position adding the following linker:

5'-CGTCGACAGTAC-3' (Seq. ID No.: 18)

15 CATGGCAGCTGT-5' (Seq. ID No.: 19)

In effect, the hLF sequence is now surrounded by two unique restriction sites (ClaI and SalI) and can be replaced by any recombinant ANA sequence that has a ClaI-site at the 5'- end and a SalI-site at the 3'- end.

20 Another transgene was constructed that is identical to the foregoing except that it contains only about 8kb of 5' $\alpha S1$ -casein expression regulation sequence. It was constructed by taking the NotI-ClaI fragment from pNE3BS and fusing it directly into Xho-NotI fragment from clone pHLF 3' 10kb. The resulting plasmid was designated pGPIhLF (7kb) (also referred to as p8.8HLF4).

Plasmid 16,8hLF4 was modified to contain a hybrid splice signal ($\alpha S1$ -casein-IgG) described in examples 3 and 5. The resulting plasmid was designated 16,8hLF3 and is 30 identical to 16,8hLF4 except for the presence of a hybrid intron versus a "natural" casein intron in the 5'-UTR.

The hLF signal sequence can also be used in all of the cDNA constructs disclosed herein instead of the casein signal sequence. This can be done in the following way: A synthetic oligo was made that contains the complete hLF signal sequence (see Fig. 2) plus a ClaI restriction site at the 5'-end and an EagI restriction site at the 3'-end. These restriction sites also border the casein-signal sequence in the other plasmids (e.g., p16,8hLF4). A fragment containing the hLF-cDNA surrounded by ClaI and SalI sites was cloned in pGEM7 (Stratagene, Inc.) containing a ClaI and SalI site. The resulting plasmid was digested with ClaI and EagI and used as a vector to accommodate the ClaI-EagI fragment containing the hLF sequence. From the positive clones, the cDNA, with its own sequence, was excised as a ClaI-SalI fragment and inserted in ClaI-SalI digested p16,8hLF4 to generate p16,8hLF5. Similarly, this Cla-Sal fragment containing the hLF-cDNA plus hLF signal sequence can be inserted in any hLF cDNA vector.

20

EXAMPLE 14Production of Recombinant Human Lactoferrin
in the Milk of Transgenic Mice

Transgenic mice were generated utilizing several of the transgenes identified in the examples herein. The transgenes used are identified in Table 3. In each case, the 5' and 3' expression regulation sequences were from the bovine α S1-casein gene, the RNA splice signal in the 5' untranslated region was either homologous from the α S1-casein gene or a hybrid casein-IgG intervening sequence. The recombinant DNA in each case was derived from cDNA clones.

TABLE 3

	Plasmid from which transgene excised	Length of 5'-expression regulator segment (kb)	Length of 3'-expression regulator segment	IVS	Number of strains	Maximum expression level recorded	Percentage of mice that have expression levels exceeding $\mu\text{g/ml}$
5	p0.7,S HLF4	0.7	8	homologous	5	5 $\mu\text{g/ml}$	0
	p8,S HLF4	8	8	homologous	6	140 $\mu\text{g/ml}$	67
	p16,S HLF4	16	8	homologous	4	8 $\mu\text{g/ml}$	0
	p16,S HLF3	16	8	hybrid	7	1330 $\mu\text{g/ml}$	43

10

EXAMPLE 15Construction of Transgene Cassette
for Genomic Recombinant DNA

The plasmids described so far all contain regions
 derived from the bovine αS1 -casein untranscribed regions
 15 (including intervening sequences). When a genomic gene
 is to be expressed that already contains untranslated
 regions and intervening sequences permissive for high
 expression, it is preferable to use expression cassettes
 where the flanking regions of the αS1 -casein gene are
 20 operably linked to the untranslated regions of the gene
 to be expressed. Such an expression cassette is p-
 16kb,CS and was constructed as follows: plasmid pS1
 3'5'hLF was used as a template in a PCR experiment.
 This plasmid contains 680 bp of promoter sequence of the
 25 αS1 -casein gene as well as its first exon. the rest of
 this plasmid is not relevant for this experiment. The
 upstream primer was located just upstream of the insert
 in the plasmid moiety (just upstream of a NotI
 restriction site). Its sequence is: 5'-CGA CGT TGT AAA
 30 ACG ACGG-3'.

The downstream primer was located in exon 1. Its
 sequence matches the first 19 bp of the exon exactly and

also has a non-hybridizing region of 17 bp containing a ClaI and a SalI site. It has the following sequence:

5'-ATTGTCGACTTATCGATGGGTTGATGATCAAGGTGA-3'

The amplified fragment was digested with NotI and SalI and ligated into pKUN2 (see Example 13). The resulting plasmid (p-680CS) therefore harbors a proximal promoter fragment from -680 to +19, plus two restriction sites just downstream of those 19 bp.

This plasmid was digested with NotI (just upstream of -680) and NsiI (at-280) and used as a vector to ligate to a fragment running from a NotI site (just upstream of -16kb) to NsiI (-280) isolated from p16,8hLF4 (Example 13). This plasmid (p-16kb,CS) therefore harbors a promoter fragment from \approx -16,000 to +19. It can be used to insert genomic genes that carry their own UTR's and poly(A)-signal. After insertion of the genomic gene as a ClaI-SalI fragment, the α S1-casein 3'-flanking region can be inserted as a SalI-fragment.

EXAMPLE 16

Construction of Transgene for Production of Protein C

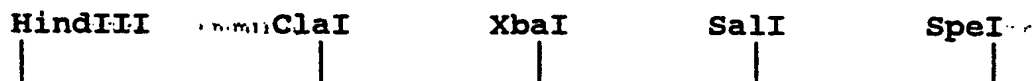
The genomic sequence of Protein C has been published. Foster, et al. (1985) Proc. Natl. Acad. Sci. USA **82**, 4673-4677. This sequence, however, does not include the first exon which was identified through the cDNA sequence published by Beckman, et al. (1985) Nucl. Acids Res. **13**, 5233-5247. The first exon of Protein C is located at position -1499 to -1448 in the Foster sequence. The transgene for expressing and secreting Protein C into the milk of bovine species is shown in Fig. 10. This transgene was constructed as follows.

A human genomic library in EMBL-3 (Clonotech) is probed with a sequence specific for protein C. A purified phage DNA prep containing the complete Protein C gene

is isolated. The phage is isolated from an E. coli strain having the Dam phenotype, such a strain GM113. This results in cloned DNA which is not methylated and as such all ClaI restriction sites can be cleaved.

- 5 A ClaI NheI fragment running from positions +1333 to 11483 is isolated. This is designated fragment I.

pGEM7 (Stratogene, Inc.) is digested with SphI and SmaI. The region in between is replaced by the corresponding region of plasmid pKUN (Gene (1986) 46, 269-276). The
10 resulting plasmid is designated pGEM7A and has the following restriction map in the relevant region:



- 15 Two primers are synthesized. Primer GP125 has the following sequence:

5' - CAA ATC GAT TGA ACT TGC AGT ATC TCC ACG AC - 3'
 ClaI

Primer GP 126 has the following sequence:

20 5' - GGG ATC GAT CAG ATT CTG TCC CCC AT - 3'
 ClaI

Primer GP125 has an overlap with exon 0 (position 654 to 675 of the Protein C gene) and introduces a ClaI site in the 5' untranslated region. Exon 0 is the exon not
25 identified by Foster, et al. Primer GP126 overlaps the region from 1344 to 1315 in the Protein C gene. This region contains a ClaI site.

The region between position 654 and 1344 is amplified using either human DNA or phage DNA as a template. The

so amplified material is digested with ClaI and cloned in vector pGEN7a to form pPCCC. This vector is propagated in a dam negative strain such as GM113 and partially cut with ClaI (only the plasmids that are cut
5 once with ClaI at position 1340 are of interest) and completely with XbaI. The ClaI NheI fragment (fragment 1) is cloned into this vector. The resultant plasmid is designated pPC. Its structure is shown in Fig. 10. From this plasmid, the Protein C transgene is isolated
10 as a ClaI-SalI fragment and ligated into p16Kb, CS (See Example 15) to generate a transgene capable of expressing Protein C in bovine milk, this plasmid is designated p16 Kb,CS,PC.

The transgene contained within plasmid p 16 Kb, CS, PC
15 is excised with NotI and used to generate transgenic bovine species as previously described. Such transgenic animals are capable of producing protein C in their milk.

Having described the preferred embodiments of the
20 present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

25 All references disclosed herein are expressly incorporated by reference.

WHAT IS CLAIMED IS:

1. A transgene for producing a recombinant polypeptide in transgenic bovine species comprising at least one expression regulation DNA sequence functional in at least one cell-type of said bovine species operably linked to a recombinant DNA encoding a recombinant polypeptide, wherein said transgene is capable of directing the expression of said recombinant DNA sequence in at least said one cell-type of a bovine species containing said transgene to produce said recombinant polypeptide.

2. The transgene of Claim 1 wherein said expression regulation sequences comprise 5' and 3' expression regulation sequences from a serum albumin, said cell-type is liver cell, said recombinant polypeptide is human serum albumin and said transgene further comprises a secretory DNA sequence functional in said liver cells and operably linked to the recombinant DNA encoding said human serum albumin.

3. A transgene for producing a recombinant polypeptide in the milk of transgenic bovine species comprising at least one expression regulation DNA sequence functional in the mammary secretory cells of said bovine species, a secretory DNA sequence encoding a secretory signal sequence also functional in the mammary secretory cells of said bovine species and a recombinant DNA sequence encoding a recombinant polypeptide, said secretory DNA sequence being operably linked to said recombinant DNA sequence and to form a secretory-recombinant DNA sequence and said at least one expression regulation sequence being operably linked to said secretory-recombinant DNA sequence, wherein said transgene is capable of directing the expression of said secretory-recombinant DNA sequence in mammary secretory

cells of bovine species containing said transgene to produce a form of recombinant polypeptide which when secreted from said mammary secretory cells produces recombinant polypeptide in the milk of said bovine species.

4. The transgene of Claim 1 or 3 further comprising a recombinant intervening sequence.

5. The transgene of Claim 4 wherein said recombinant intervening sequence is a hybrid intervening sequence.

6. The transgene of Claim 5 wherein said hybrid intervening sequence contains a permissive RNA splice signal.

7. The transgene of Claim 3 wherein said recombinant polypeptide is a homologous polypeptide from bovine species.

8. The transgene of Claim 7 wherein said homologous polypeptide is selected from the group consisting of caseins, lactoferrin, lysozyme, cholesterol hydrolase and serum albumin.

9. The transgene of Claim 3 wherein said recombinant polypeptide is a heterologous polypeptide.

10. The transgene of Claim 9 wherein said heterologous polypeptide is selected from the group consisting of human milk proteins, human serum proteins, and industrial enzymes.

11. The transgene of Claim 10 wherein said heterologous polypeptide is a human milk protein.

12. The transgene of Claim 11 wherein said human milk protein is selected from the group consisting of secretory immunoglobulins, lysozyme, lactoferrin, lactoglobulin, α -lactalbumin and bile salt-stimulated lipase.

13. The transgene of Claim 12 wherein said milk protein is lactoferrin.

14. The transgene of Claim 10 wherein said heterologous polypeptide is a human serum protein.

15. The transgene of Claim 14 wherein said human serum protein is selected from the group consisting of albumin, immunoglobulin, Factor VIII, Factor IX and Protein C.

16. The transgene of Claim 15 wherein said serum protein is albumin.

17. The transgene of Claim 10 wherein said heterologous polypeptide is an industrial enzyme selected from the group consisting of proteases, lipases, chitinases and ligninases.

18. The transgene of Claim 3 wherein said secretory DNA sequence is selected from the group consisting of DNA sequences encoding secretory signal sequences from human lactoferrin, human serum albumin, and secretory signal sequences from bovine α S1-casein, α S2-casein, β -casein, κ -casein, α -lactalbumin, β -lactoglobulin, and serum albumin.

19. The transgene of Claim 18 wherein said secretory DNA sequence is the DNA sequence encoding the signal secretion sequence of bovine α S1 casein.

20. The transgene of Claim 3 wherein said at least one expression regulation sequence comprises 5' expression regulation DNA sequences, wherein said 5' expression regulation DNA sequence is operably linked to the 5' end of said secretory-recombinant DNA sequence.

21. The transgene of Claim 20 wherein said 5' expression regulation DNA sequence is selected from the group consisting of 5' expression regulation sequence from bovine genes encoding α S1-casein, α S2-casein, β -casein, κ -casein, α -lactalbumin, and β -lactoglobulin.

22. The transgene of Claim 21 wherein said 5' expression regulation DNA sequence is a proximal 5' expression regulation sequence comprising the promoter of bovine α S1-casein.

23. The transgene of Claim 22 wherein said 5' expression regulation DNA sequence further comprises a distal 5' expression regulation sequence comprising 5'-flanking DNA sequence from bovine α S1-casein.

24. The transgene of Claim 20 further comprising 3' expression regulation sequences operably linked to the 3' end of said secretory-recombinant DNA sequence.

25. The transgene of Claim 24 wherein said 3' expression regulation sequence comprise 3' expression regulation sequence from bovine genes encoding α S1-casein, α S2-casein, β -casein, κ -casein, α -lactalbumin, and β -lactoglobulin.

26. The transgene of Claim 25 wherein said 3' expression regulation DNA sequence comprises a 3' proximal expression regulation sequence from bovine α S1-casein.

27. The transgene of Claim 26 wherein said 3' expression regulation DNA sequence further comprises a 3' distal expression regulation sequence from bovine α S1-casein.

5 28. The transgene of Claim 27 wherein said distal 5' expression regulation DNA sequence comprises about a 30kb 5'-flanking region of bovine α S1-casein and said distal 3' expression regulation DNA sequence comprises about a 15kb 3'-flanking region of bovine α S1-casein.

10 29. A transgenic bovine species capable of producing a recombinant polypeptide in at least one cell type of said animal.

15 30. A transgenic bovine species capable of producing recombinant polypeptide in the milk of said transgenic species.

31. The transgenic bovine species of Claim 30 wherein said recombinant polypeptide is a homologous polypeptide from bovine species.

20 32. The transgenic bovine species of Claim 30 wherein said recombinant polypeptide is a heterologous polypeptide.

25 33. The transgenic bovine species of Claim 30 wherein said heterologous polypeptide is selected from the group consisting of human milk proteins, human serum proteins, and industrial enzymes.

34. The transgenic bovine species of Claim 33 wherein said heterologous polypeptide is a human milk protein.

35. The transgenic bovine species of Claim 34 wherein said human milk protein is selected from the group

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consisting of secretory immunoglobulins, lysozyme, lactoferrin, lactoglobulin, α -lactalbumin and bile salt-stimulated lipase.

36. The transgenic bovine species of Claim 35 wherein said milk protein is lactoferrin.

37. The transgenic bovine species of Claim 33 wherein said heterologous polypeptide is a human serum protein.

38. The transgenic bovine species of Claim 37 wherein said human serum protein is selected from the group consisting of albumin, immunoglobulin, Factor VIII, Factor IX and Protein C.

39. The transgenic bovine species of Claim 38 wherein said serum protein is albumin.

40. The transgenic bovine species of Claim 33 wherein said heterologous polypeptide is an industrial enzyme selected from the group consisting of proteases, lipases, chitinases and ligninases.

41. Milk from transgenic bovine species containing a recombinant polypeptide.

42. The milk of Claim 41 wherein said recombinant polypeptide is a homologous polypeptide from bovine species.

43. The milk of Claim 41 wherein said recombinant polypeptide is a heterologous polypeptide.

44. The milk of Claim 43 wherein said heterologous polypeptide is selected from the group consisting of human milk proteins, human serum proteins, and industrial enzymes.

45. The milk of Claim 44 wherein said heterologous polypeptide is a human milk protein.

46. The milk of Claim 45 wherein said human milk protein is selected from the group consisting of secretory immunoglobulins, lysozyme, lactoferrin, lactoglobulin, α -lactalbumin and bile salt-stimulated lipase.

47. The milk of Claim 46 wherein said milk protein is lactoferrin.

48. The milk of Claim 43 wherein said heterologous polypeptide is a human serum protein.

49. The milk of Claim 48 wherein said human serum protein is selected from the group consisting of albumin, immunoglobulin, Factor VIII, Factor IX and Protein C.

50. The milk of Claim 49 wherein said serum protein is albumin.

51. A food formulation comprising transgenic milk containing a recombinant polypeptide.

52. The food formulation of Claim 51 wherein said recombinant polypeptide is at least partially purified from said transgenic milk.

53. The food formulation of Claim 31 formulated with nutrients appropriate for infant formula.

54. A method for producing a transgenic bovine species capable of producing a recombinant polypeptide in the milk of said bovine species, said method comprising:

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introducing the transgene of Claim 1 into an embryonal target cell of a bovine species;

transplanting the transgenic embryonal target cell formed thereby or the embryo obtained herefrom into a recipient female bovine parent; and

identifying at least one female offspring which is capable of producing said recombinant polypeptide in the milk of said offspring.

55. A method for producing a transgenic non-human mammal having a desirable phenotype comprising:

(a) methylating a transgene capable of conferring said phenotype when incorporated into the cells of said transgenic non-human animal;

(b) introducing said methylated transgene into fertilized oocytes of said non-human mammal to permit integration of said transgene into the genomic DNA of said fertilized oocytes;

(c) culturing the individual oocytes formed hereby to pre-implantation embryos thereby replicating the genome of each of said fertilized oocytes;

(d) removing at least one cell from each of said pre-implantation embryos and lysing said at least one cell to release the DNA contained therein;

(e) contacting said released DNA with a restriction endonuclease capable of cleaving said methylated transgene but incapable of cleaving the unmethylated form of said transgene formed after integration into and replication of said genomic DNA; and

(f) detecting which of said cells from said pre-implantation embryos contain a transgene which is resistant to cleavage by said restriction endonuclease as an indication of which pre-implantation embryos have integrated said transgene.

56. The method of Claim 55 wherein said removal of at least one cell forms a first and second hemi-embryo

for each of said pre-implementation embryos and each of said first hemi-embryos is lysed and analyzed according to steps (d) through (f), said method further comprising;

5 (g) cloning at least one of said second hemi-embryos; and

(h) to form a multiplicity of transgenic embryos.

10 57. The method of Claim 56 further comprising transplanting more than one of said transgenic embryos into recipient female parents to produce a population containing at least two transgenic non-human animals having the same genotype.

15 58. The method of Claim 55 further comprising transplanting the remainder of said pre-implantation embryo containing a genomically integrated transgene into a recipient female parent and identifying at least one offspring having said phenotype.

20 59. The method of Claim 55 wherein said restriction endonuclease is DPNI and said transgene is methylated at N6 of the adenine of the sequence GATC contained within said transgene.

25 60. The method of Claim 59 wherein said detection utilizes a polymerase chain reaction using extension primers complementary to sequences upstream and downstream to said GATC sequence.

30 61. The method of Claim 59 wherein said non-human transgenic mammal is bovine species, said transgene encodes a recombinant polypeptide and said desired phenotype is the ability to produce said recombinant polypeptide in the milk of said bovine species.

62. The method of Claim 61 wherein said transgene is the transgene of Claim 3.

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sau961
 haeIII
 asuI
 sau961
 nlaIV
 hgiI
 ecoRI
 bsp1286
 banII
 asuI
 nlaIV
 mnlI
 mboII
 auaI
 apaI
 mnlI
 mnlI
 ecoRI
 tth1111
 1 GGA CTT GTC TTC CTC GTC CTG CTG TTC CTC GGG GCC CTC GGA CTG
 -18 Gly Leu Val Phe Leu Val Leu Leu Phe Leu Gly Ala Leu Gly Leu
 haeIII
 eaeI
 cfrI
 hinPI
 hhaI
 46 TGT CTG GCT GGC CGT AGG AGA AGG AGT GTT CAG TGG TGC GCC GTA TCC
 -3 Cys Leu Ala Gly Arg Arg Arg Arg Ser Val Gln Trp Cys Ala Val Ser
 haeIII
 mnlI
 auaI
 haeI
 94 CAA CCC GAG GCC ACA AAA TCG TTC CAA TGG CAA AGG AAT ATG AGA AAA
 14 Gln Pro Glu Ale Thr Lys Cys Phe Gln Trp Gln Arg Asn Met Arg Lys
 mnlI
 sau961
 haeIII
 asuI
 fnu4HI
 bbvI
 aluI
 pvuII
 pleI
 hinfI
 bsrI
 bsmI
 fokI
 142 GTG CTG GGC CCT CCT GTC AGC TGC ATA AAG AGA GAC TCC CCC ATC CAG
 30 Val Arg Gly Pro Pro Val Ser Cys Ile Lys Arg Asp Ser Pro Ile Gln
 haeIII
 haeI
 scrFI
 ecoRI
 bstNI
 haeIII
 sau961
 asuI
 sfaNI
 190 TGT ATC CAG GCC ATT GCG GAA AAC AGG GCC GAT GCT GTG ACC CTT GAT
 46 Cys Ile Gln Ale Ile Ala Glu Asn Arg Ala Asp Ala Val Thr Leu Asp
 sau961
 nlaIV
 scrFI
 ecoRI
 bstNI
 haeIII
 stuI
 haeIII
 mnlI
 haeI
 asuI
 238 GGT GGT TTC ATA TAC GAG GCA GGC CTG GCC CCC TAC AAA CTG CGA CCT
 62 Gly Gly Phe Ile Tyr Glu Ala Gly Leu Ala Pro Tyr Lys Leu Arg Pro

FIG.-1A

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sau961
avaII
asuI
fnu4HI accI nlaIV

286 GTA GCG GCG GAA GTC TAC GGG ACC GAA AGA CAG CCA CGA ACT CAC TAT
78 Val Ala Ala Glu Val Tyr Gly Thr Glu Arg Gln Pro Arg Thr His Tyr

fnu4HI mboII bbvI aluI
hphI fnu4HI aluI pvuII

334 TAT GCC GTG GCT GTG GTG AAG AAG GGC GGC AGC TTT CAG CTG AAC GAA
94 Tyr Ala Val Ala Val Val Lys Lys Gly Gly Ser Phe Gln Leu Asn Glu

haeIII sau961
stuI avaII
bglI haeI asuI fokI

382 CTG CAA GGT CTG AAG TCC TGC CAC ACA GGC CTT CGC AGG ACC GCT GGA
110 Leu Gln Gly Leu Lys Ser Cys His Thr Gly Leu Arg Arg Thr Ala Gly

sau961
avaII
asuI
nlaIV

430 TGG ATT GTC CCT ACA GGG ACA CTT CGT CCA TTC TTG AAT TGG ACG GTT
126 Trp Asn Val Pro Thr Gly Thr Leu Arg Pro Phe Leu Asn Trp Thr Gly

hgiIII aluI
bsp1286 fnu4HI
banII bbvI ddeI aluI
ddeI mnlI pvuII mboII pvuII

478 CCA CCT GAG CCC ATT GAG GCA GCT GTG CAG TTC TTC TCA GCC AGC TGT
142 Pro Pro Glu Pro Ile Glu Ala Ala Val Gln Phe Phe Ser Ala Ser Cys

mspI
hpaII
scrFI
ncI
cauII

526 GTT CCC GGT GCA GAT AAA GGA CAG TTC CCC AAC CTG TGT CGC CTG TGT
158 Val Pro Gln Ala Asp Lys Gly Gln Phe Pro Asn Leu Cys Arg Leu Cys

nlaIV
scrFI
ecoRII
mnlI bstNI rsal

574 GCG GGG ACA GGG GAA AAC AAA TGT GCC TTC TTC TTC CAG GAA CCG TAC
174 Ala Gly Thr Gly Glu Asn Lys Cys Ala Phe Ser Ser Gln Glu Pro Tyr

FIG.-1B

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	alul	nlaIV hgiCI	banI	ddeI	bsmaI	bsmaI
622	TTC AGC TAC TCT	GGT GCC TTC AAG	TGT CTG AGA GAC	GGG GCT GGA	GAC	
190	Phe Ser Tyr Ser	Gly Ala Phe Lys	Cys Leu Arg Asp	Gly Ala Gly	Asp	

				sau961		
				avaII		
				asuI		
				ppuMI		
		hgiAI		ecoD109I		
		bsp1286		mnII		mnII
670	GTG GCT TTT ATC	AGA GAG AGC ACA	GTG TTT GAG	GAC CTG TCA	GAC GAG	
206	Val Ala Phe Ile	Arg Glu Ser Thr	Val Phe Glu	Asp Leu Ser	Asp Glu	

718	GCT GAA AGG GAC	GAG TAT GAG TTA	CTC TGC CCA	GAC AAC ACT	CGG AAG	
222	Ala Glu Arg Asp	Glu Tyr Glu	Leu Leu Cys	Pro Asp Asn	Ser Arg Lys	

				scrFI		
				ncII		
				mspI		
				hpaII		
				cauII		
				xmaI sau961		
				smaI nlaIV		
				scrFI		
				ncII avaII		
				cauII		
				avaI asuI		
				sau961 ppuMI		
				haeIII nlaIV		
	bsrI			asuI	ecoD109I	nlaIII
766	CCA GTG GAC AAG	TTC AAA GAC TGC	CAT CTG GCC	CGG GTC CCT	TCT CAT	
238	Pro Val Asp Lys	Phe Lys Asp Cys	His Leu Ala	Arg Val Pro	Ser His	

				sfaNI		
				fokI		mbolI
	bgII	draIII		mnII		hinfI
814	GCC GTT GTG GCA	CGA AGT GTG AAT	GGC AAG GAG	GAT GCC ATC	TGG AAT	
254	Ala Val Val Ala	Arg Ser Val Asn	Gly Lys Glu	Asp Ala Ile	Trp Asn	

				scrFI		
				ecoRII		
				bstNI		
						hphI
862	CTT CTC CGC CAG	GCA CAG GAA AAG	TTT GGA AAG	GAC AAG TCA	CCG AAA	
270	Leu Leu Arg Gln	Ala Gln Glu	Lys Phe Gly	Lys Asp Lys	Ser Pro Lys	

FIG.-1C

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sau3AI
 mboI
 dpnI
 xhoII
 bstYI
 bglII
 aluI
 bstXI
 nlaIV
 910 TTC CAG CTC TTT GGC TCC CCT AGT GGG CAG AAA GAT CTG CTG TTC AAG
 286 Phe Gln Leu Phe Gly Ser Pro Ser Gly Gln Lys Asp Leu Leu Phe Lys

nlaIV
 hgiCI
 pleI
 hinfI
 mnlI bsp1286 mnlI
 taqI banI auaI hinfI
 958 GAC TCT GCC ATT GGG TTT TCG AGG GTG CCC CCG AGG ATA GAT TCT GGG
 302 Asp Ser Ala Ile Gly Phe Ser Arg Val Pro Pro Arg Ile Asp Ser Gly

mspI
 hpaII
 styl
 rsaI nlaIV foki mnlI-
 1006 CTG TAC CTT GGC TCC GGC TAC TTC ACT GCC ATC CAG AAC TTG AGG AAA
 318 Leu Tyr Leu Gly Ser Gly Tyr Phe Thr Ala Ile Gln Asn Leu Arg Lys

mspI
 hpaII
 scrFI
 nciI
 mnlI
 fnu4HI
 bbvI couII
 hinfI
 hhaI
 1054 AGT GAG GAG GAA GTG GCT GCC CGG CGT GCG CGG GTC GTG TGG TGT GCG
 344 Ser Glu Glu Glu Val Ala Ala Arg Arg Ala Arg Val Val Trp Cys Ala

hinfI
 mstI
 fspI
 fnu4HI
 aluI hhaI
 bstXI
 bsrI
 alwNI bbvI
 1102 GTG GGC GAG CAG GAG CTG CGC AAG TGT AAC CAG TGG AGT GGC TTG AGC
 350 Val Gly Glu Gln Glu Leu Arg Lys Cys Asn Gln Trp Ser Gly Leu Ser

fnu4HI
 bbvI
 bspMI
 mnlI haeIII
 mnlI
 sfaNI
 1150 GAA GGC AGC GTG ACC TGC TCC TCG GCC TCC ACC ACA GAG GAC TGC ATC
 366 Glu Gly Ser Val Thr Cys Ser Ser Ala Ser Thr Thr Glu Asp Cys Ile

scrFI
 ecoRII
 bstNI
 bstXI
 aluI sfaNI nlaIII foki mnlI
 1198 GCC CTG GTG CTG AAA GGA GAA GCT GAT GCC ATG AGT TTG GAT GGA GGA
 382 Ala Leu Val Leu Lys Gly Glu Ala Asp Ala Met Ser Leu Asp Gly Gly

FIG.-1D

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		nlaIII		nlaIV		scrFI
		sphI		hgiCI		ecoRII
	rsaI	nspCI		banI		bstNI
1246	TAT GTG TAC ACT	GCA TGC AAA TGT	GGT TTG GTG CCT	GTC CTG GCA GAG		
398	Tyr Val Tyr Thr	Ala Cys Lys Cys	Gly Leu Val Pro	Val Leu Ala Glu		
				sau3AI		
				mbol		
				dpnI		
				alwI		
1294	AAC TAC AAA TCC	CAA CAA AGC AGT	GAC CCT GAT CCT	AAC TGT GTG GAT		
414	Asn Tyr Lys Ser	Gln Gln Ser Ser	Asp Pro Asp Pro	Asn Cys Val Asp		
				sau3AI		
				mbol		
				dpnI		
	ecoNI	ecoRV				
1342	AGA CCT GTG GAA	GGA TAT CTT GCT	GTG GCG GTG GTT	AGG AGA TCA GAC		
430	Arg Pro Val Glu	Gly Tyr Leu Ala	Val Ala Val Val	Arg Arg Ser Asp		
				scrFI		
				ecoRII		
				bstNI		
1390	ACT AGC CTT ACC	TGG AAC TCT GTG	AAA GGC AAG AAG	TCC TGC CAC ACC		
446	Thr Ser Leu Thr	Trp Asn Ser Val	Lys Gly Lys Lys	Ser Cys His Thr		
				haeIII		
				nlaIII		
				styI sau96I		mbol
				ncol asuI		earI
		pstI				
1438	GCC GTG GAC AGG	ACT GCA GGC TGG	AAT ATC CCC ATG	GGC CTG CTC TTC		
462	Ala Val Asp Arg	Thr Ala Gly Trp	Asn Ile Pro Met	Gly Leu Leu Phe		
				nlaIV		
				hgiIII		
				bsp1286		
				banII		
				sspl		alul bsp1286
1486	AAC CAG ACG GGC	TCC TGC AAA TTT	GAT GAA TAT TTC	AGT CAA AGC TGT		
478	Asn Gln Thr Gly	Ser Cys Lys Phe	Asp Glu Tyr Phe	Ser Gln Ser Cys		
				sau3AI		
				mbol		
				DpnI		
				xhoII		
	scrFI			bstYI		
	ecoRII			hgiAI		
	bstNI	avaI	bgIII	bsp1286		
1534	GCC CCT GGG TCT	GAC CCG AGA TCT	AAT CTC TGT GCT	CTG TGT ATT GGC		
484	Ala Pro Gly Ser	Asp Pro Arg Ser	Asn Leu Cys Ala	Leu Cys Ile Gly		

FIG.-1E

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hphI 4/25 bsp1286

1582 GAC GAG CAG GGT GAG AAT AAG TGC GTG CCC AAC AGC AAT GAG AGA TAC
 510 Asp Glu Gln Gly Glu Asn Lys Cys Val Pro Asn Ser Asn Glu Arg Tyr

nlaIV
 hgiCI
 banI scrFI
 mspI ecoRII
 bsrI hpaII bstNI ddeI bsmI bsmal

1630 TAC GGC TAC ACT GGG GCT TTC CGG TGC CTG GCT GAG AAT GCT GGA GAC
 526 Tyr Gly Tyr Thr Gly Ala Phe Arg Cys Leu Ala Glu Asn Ala Gly Asp

1678 GTT GCA TTT GTG AAA GAT GTC ACT GTC TTG CAG AAC ACT GAT GGA AAT
 542 Val Ala Phe Val Lys Asp Val Thr Val Leu Gln Asn Thr Asp Gly Asn

fnu4HI
 bbvI
 hlnFI
 hhaI

mnlI nlaIII ddeI aluI

1726 AAC AAT GAG GCA TGG GCT AAG GAT TTG AAG CTG GCA GAC TTT GCG CTG
 558 Asn Asn Glu Ala Trp Ala Lys Asp Leu Lys Leu Ala Asp Phe Ala Leu

taqI
 mnlI
 bglI

fnu4HI
 bbvI
 aluI

ddeI

1774 CTG TGC CTC GAT GGC AAA CGG AAG CCT GTG ACT GAG GCT AGA AGC TGC
 574 Leu Cys Leu Asp Gly Lys Arg Lys Pro Val Thr Glu Ala Arg Ser Cys

sau96I
 nlaIV
 nlaIII
 styI haeIII
 ncoI asuI hinfI nlaIII bsmal fokI

1822 CAT CTT GCC ATG GCC CCG AAT CAT GCC GTG GTG TCT CGG ATG GAT AAG
 590 His Leu Ala Met Ala Pro Asn His Ala Val Val Ser Arg Met Asp Lys

fnu4HI
 ecoNI alwNI bbvI

1870 GTG GAA CGC CTG AAA CAG GTG CTG CTC CAC CAA CAG GCT AAA TTT GGG
 606 Val Glu Arg Leu Lys Gln Val Leu Leu His Gln Gln Ala Lys Phe Gly

sau3AI
 mboI mspI
 dpnI hpaII
 xhoII scrFI
 bstYI nciI
 alwI cauII

bsrI

1919 AGA AAT GGA TCT GAC TGC CCG GAC AAG TTT TGC TTA TTC CAG TCT GAA
 622 Arg Asn Gly Ser Asp Cys Pro Asp Lys Phe Cys Leu Phe Gln Ser Glu

FIG.-1F

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                                haeIII
                                haeI
                                eaeI          styl
                                ddeI          cfrI      pleI ncoI
                                draIII       balI      hinfI
1966 ACC AAA AAC CTT CTG TTC ATT GAC AAC ACT GAG TGT CTG GCC AGA CTC
638 Thr Lys Asn Leu Leu Phe Asn Asp Asn Thr Glu Cys Leu Ala Arg Leu

                                sau961
                                avall
                                asuI
                                mlaIII      ndeI      sspI      nlaIV
2014 CAT GGC AAA ACA ACA TAT GAA AAA TAT TTG GGA CCA CAG TAT GTC GCA
654 His Gly Lys Thr Thr Tyr Glu Lys Tyr Leu Gly Pro Gln Tyr Val Ala

                                scrFI
                                ecoRII
                                bstNI
                                hgiAI
                                bsp1286      mnlI      mnlI
2062 GGC ATT ACT AAT CGT AAA AAG TGC TCA ACC TCC CCC CTC CTG GAA GCC
670 Gly Ile Thr Asn Leu Lys Lys Cys Ser Thr Ser Pro Leu Leu Glu Ala

                                ddeI
                                nstII
                                mnlI
                                eco8II
                                ecoRI      bsu36I
2110 TGT GAA TTC CTC AGG AAG TAA AACC GAAGAA GATGGCCAG CTCCCAAGA
685 Cys Glu Phe Leu Arg Lys DC*

                                styl
                                haeIII
                                sau961
                                mboII      scrFI      asuI
                                earI      ecoRII      nlaIV
                                ddeI      mnlI      aluI      bstNI      ecoD109I nlaIV
2161 AAGCCTCAGC CATTCACTGC CCCAGCTCT TCTCCCAGG TGTGTTGGGG CCTTGGCTCC

                                ecoNI      fokI      ddeI
2221 CCTGCTGAAG GTGGGGATTG CCCATCCATC TGCTTACAAT TCCCTGCTGT CGTCTTAGCA

2281 AGAAGTAAAA TGAGAAATTT TGTGTGATATT CAAAAAAAAA

>LENGTH: 2319

```

FIG.-1G

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1 GACTCCTAGG GGCTTGACAG CCTAGTGGGA GAGAAAGAAC ATCGACAGAG CCAGGCAGAA CCAGGCAGG TGAGGTGCAG GCTGGCTTTC CTCTGCAGC
 101 GCGGTGTGGA GTCTGTCTCT GCCTCAGGCG TTTTCGGAGC CTGGATCCTC AAGGAACAAG TAGACCTGGC CGCGGGGAGT GGGGAGGAA GGGGTGTCTA
 201 TTGGCAACA GGGCGGCAAA GCCCTGAATA AAGGGGCGCA GGGCAGGCGC AAGTGACAG CCTTCGTTTG CCAAGTCGCC TCCAGACCGC AGAC ATG AAA CTT
 -19 M K L
 304 GTC TTC CTC CTG CTG TTC CTC GGG GCC CTC GGA CTG TGT CTG GCT GGC CGT AGG AGA AGG AGT GTT CAG TGG TGC GCC GTA TCC
 -16 V F L V L L F L G A L G L C L A G R R S V Q W C A V S
 391 CAA CCC GAG GCC ACA AAA TGC TTC CAA TGG CAA AGG AAT ATG AGA AAA GTG CGT GGC CCT CCT GTC AGC TGC ATA AAG AGA GAC TCC
 14 Q P E A T K C F Q W Q R N M R K V R G P V S C I K R D S
 478 CCC ATC CAG TGT ATC CAG GCC ATT GCG GAA AAC AGG GCC GAT GCT GTG ACC CTT GAT GGT TTC ATA TAG GAG GCA GGC CTG GCC
 43 P I Q C I Q A I A E N R A D A V T L D G G F I Y E A G L A
 565 CCC TAC AAA CTG CGA CCT GTA GCG GCG GAA GTC TAC GGG ACC GAA AGA CAG CCA CGA ACT CAC TAT TAT GGC GTG GCT GTG GTG AAG
 72 P K Y L R P V A A E V Y G T E R Q P R T H Y Y A V A V V K
 652 AAG GGC AGC TTT CAG CTG AAC GAA CTG CAA GGT CTG AAG TCC TGC CAC ACA GGC CTT CGC AGG ACC GCT GGA TGG AAT GTC CCT
 101 K G G S F Q L N E L Q G L K S C H T G L R R T A G W N V P
 739 ACA GGG ACA CTT CGT CCA TTC TTG AAT TGG ACG GGT CCA CCT GAG CCC ATT GAG GCA GCT GTG GCC AGG TTC TTC TCA GCC AGC TGT
 130 T G T L R P F L N V T G P E P I E A A V A R F S A S C
 826 GTT CCC GGT GCA GAT AAA GGA CAG TTC CCC AAC CTG TGT CGC CTG TGT GCG GGG ACA GGG GAA AAC AAA TGT GGC TTC TCC TCC CAG
 159 V P G A D K G Q F P N L C R L C A G T G E N K C A F S S Q
 913 GAA CCG TAC TTC AGC TAC TCT GGT GCC TTC AAG TGT CTG AGA GAC GGG GCT GGA GAC GTG GCT TTT ATC AGA GAG AGC ACA GTG TTT
 188 E P Y F S Y S G A F K C L R D G A G D V A F I R E S T V F
 1000 GAG GAC CTG TCA GAC GAG CCT GAA AGG GAC GAG TAT GAG TTA CTC TGC CCA GAC AAC ACT CGG AAG CCA GTG GAC AAG TTC AAA GAC
 217 E D L S D E A E R D E Y E L L C P D N T R K P V D K F K D
 1087 TGC CAT CTG GCC CGG GTC CCT TCT CAT GCC GTT GTG GCA CGA AGT GTG AAT GGC AAG GAG GAT GCC ATC TGG AAT CTT CTC CGC CAG
 246 C H L A R V P S H A V V A R S V N G K E D A I V N L L R Q
 1174 GCA CAG GAA AAG TTT GGA AAG GAC AAG TCA CCG AAA TTC CAG CTC TTT GGC TCC CCT AGT GGG CAG AAA GAT CTG CTG TTC AAG GAC
 275 A Q E K F G K D K S P K F Q L F G S P S G Q K D L L F K D
 1261 TCT GCC ATT GGG TTT TCG AGG GTG CCC CCG AGG ATA GAT TCT GGG CTG TAC CTT GGC TCC GGC TAC TTC ACT GCC ATC CAG AAC TTG
 304 S A I G F S R V P P R I D S G L Y L G S G Y F T A I Q N L
 1348 AGG AAA AGT GAG GAG GAA GTG GCT GCC CGG CGT GCG GTC GTG TGG TGT GCG GTG GGC GAG CAG GTG CGC AAG TGT AAC CAG
 333 R K S E E V A A R R A R V V C A V G E Q E L R K C N Q

FIG.-2A

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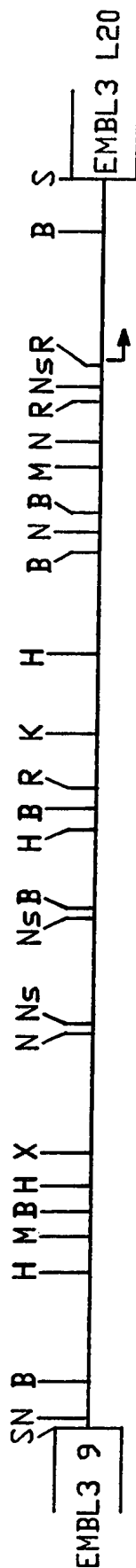
1435 TGG AGT GGC TTG AGC GAA GGC AGC GTG ACC TGC TCC GGC TCC ACC ACA GAG GAC TGC ATC GCC CTG GTG CTG AAA GGA GAA GCT
 362 V S G L S E G S V T C S S A S T T E D C I A L V L K G E A
 1522 GAT GCC ATG AGT TTG GAT GGA GGA TAT GTG TAC ACT GCA TGC AAA TGT GGT TTG GTG CCT GTC CTG GCA GAG AAC TAC AAA TCC CAA
 391 D A M S L D G G Y V Y T A C K C G L V P V L A E N Y K S Q
 1609 CAA AGC AGT GAC CCT AAT CCT AAC TGT GTG GAT AGA CCT GTG GAA GGA TAT CTT GCT GTG GCG GTG GTT AGG AGA TCA GAC ACT AGC
 420 Q S S D P D P N C V D R P V E G Y L A V A V R S D T S
 1696 CTT ACC TGG AAC TCT GTG AAA GGC AAG AAC TCC TGC GAC ACC GGC GTG GAC AGG ACT GCA GGC TGG AAT ATC CCC ATG GGC CTG CTC
 449 L T V N S V K G K S C N T A V D R T A G V N I P M Q L L
 1783 TTC AAC CAG ACG GGC TCC TGC AAA TTT GAT GAA TAT TTC AGT CAA AGC TGT GGC CCT GGG TCT GAC CCG AGA TCT AAT CTC TGT GCT
 478 F N Q T G S C K F D E Y F S C A P G S D P R S N L C A
 1870 CTG TGT ATT GGC GAC GAG GGT GAG AAT AAG TGC GTG CCC AAC AGC AAC GAG AGA TAC TAC GGC TAC ACT GGG GCT TTC CGG TGC
 507 L C I G D E Q G E N K C V P N S N E R Y Y G Y T G A F R C
 1957 CTG GCT GAG AAT GCT GGA GAC GTT GCA TTT GTG AAA GAT GTC ACT GTC TTG CAG AAC ACT GAT GGA AAT AAC AAT GAG GCA TGG GCT
 536 L A E N A G D V A F V K D V T V L Q N T D G N N E A V A
 2044 AAG GAT TTG AAC CTG GCA GAC TTT GCG CTG CTG TGC CTG GAT GGC AAA CGG AAG CCT GTG ACT GAC GCT AGA AGC TGC GAT CTT CCC
 565 K D L K L A D F A L C L D G K R K P V T E A R S C H L A
 2131 ATG GCC CCG AAT CAT GCC GTG GTG TCT CCG ATG GAT AAG GTG GAA CGC CTG AAA CAG GTG CTG CTC CAC CAA CAG GCT AAA TTT GGG
 594 M A P N H A V V S R M D K V E R L K Q V L L H Q Q A K F G
 2218 AGA AAT GGA TCT GAC TGC CCG GAC AAG TTT TGC TTA TTC CAG TCT GAA ACC AAA AAC CTT CTG TTC AAT GAC AAC ACT GAG TGT CTG
 623 R N G S D C P D K F C L F Q S E T K N L L F N D N T E C L
 2305 GCC AGA CTC CAT GGC AAA ACA ACA TAT GAA AAA TAT TTG GGA CCA CAG TAT GTC GCA GGC ATT ACT AAT CTG AAA AAG TGC TCA ACC
 652 A R L H G K T T Y E K Y L G P Q Y V A G I T N L K K C S T
 2392 TCC CCC CTC CTG GAA GCC TGT GAA TTC CTC AGG AAG TAA AACCGAAGAA GATGGCCAG CTCCCAAGA AAGCCTCAGC CATTCACTGC CCCAGCTCT
 681 S P L L E A C E F L R K D
 2491 TCTCCCCAGG TGTGTTGGG CCTGGCTCC CCTGCTGAAG GTGGGGATTG CCCATCCATC TGCTTACAAT TCCCTGCTGT CGTCTTAGCA AGAAGTAAAA
 2591 TGAGAAATTT TGTGATATT CAAAAAAA

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FIG.-2B

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RESTRICTION MAP OF 5' - FLANKING REGION OF α S1 CASEIN GENE

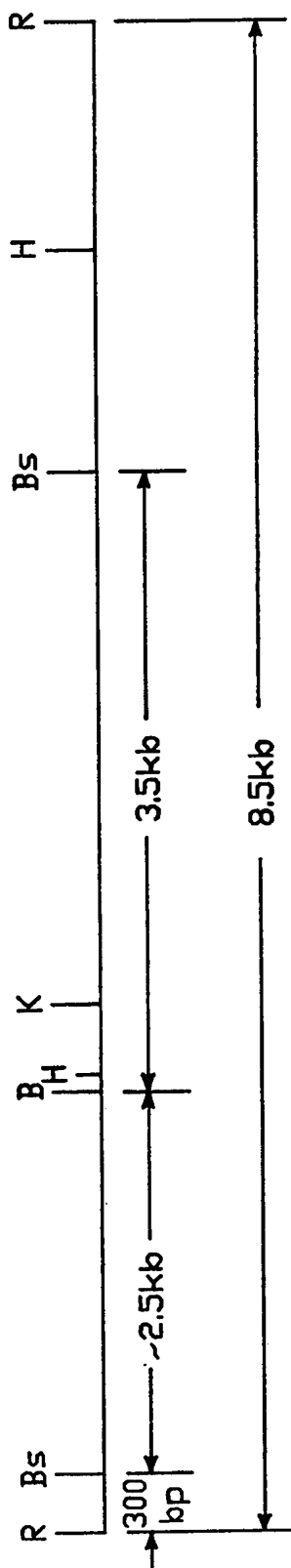


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FIG.-3

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RESTRICTION MAP OF 3' FLANKING
REGION OF α S1 CASEIN GENE



R - EcoRI
Bs - BstEI
B - BamHI
H - HindIII
K - KpnI

1kb

FIG.-4

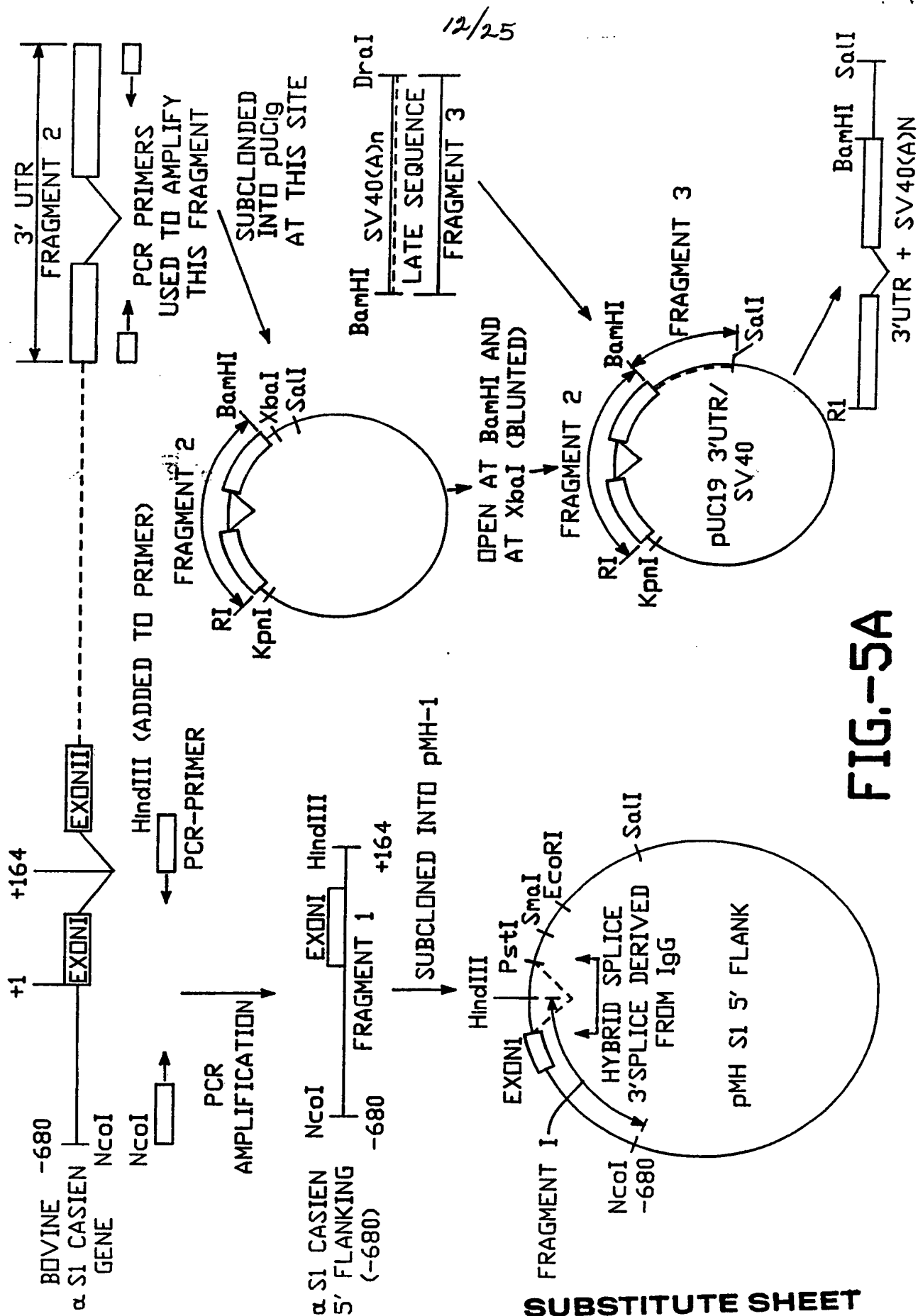
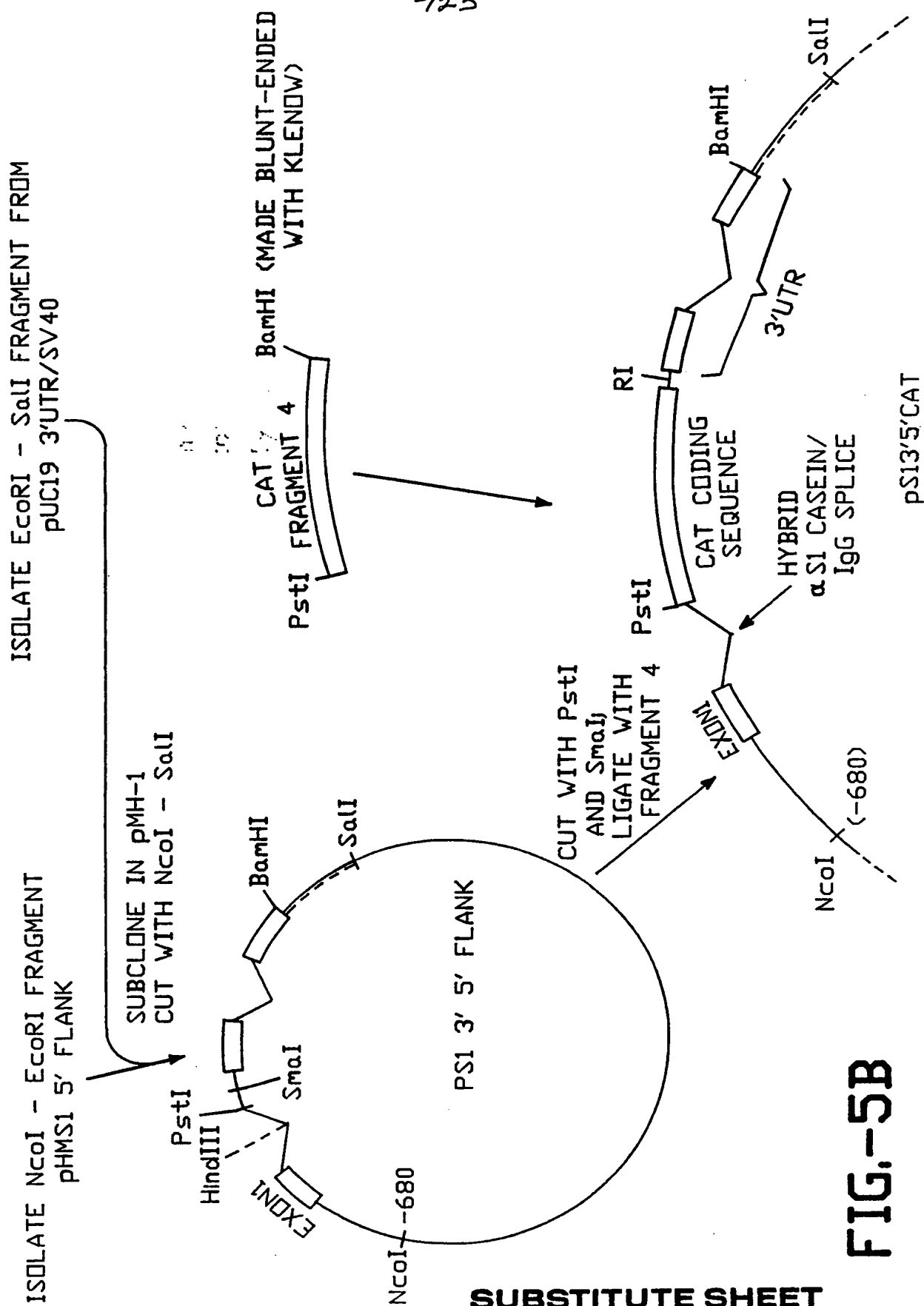


FIG.-5A

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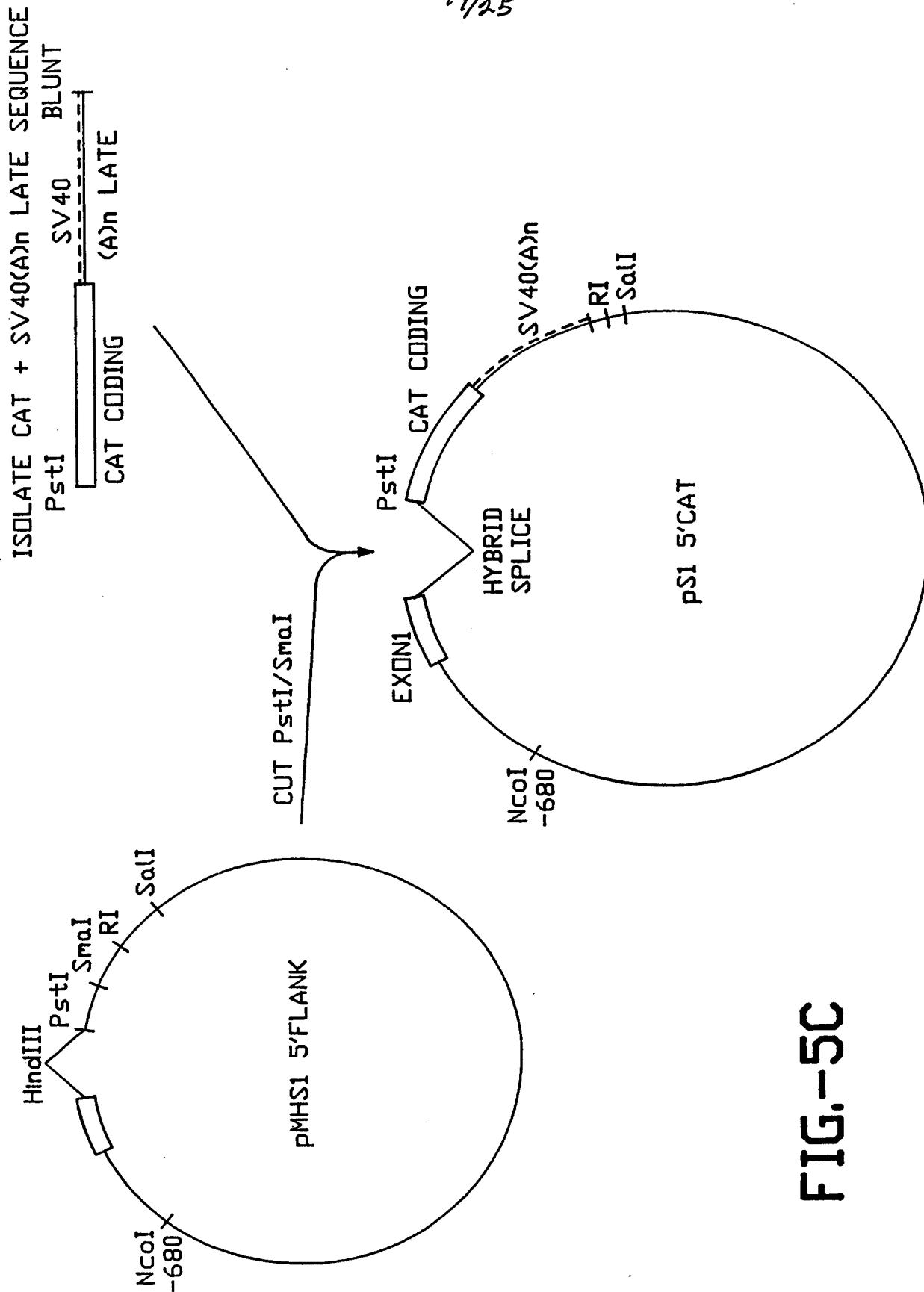


FIG.-5C

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NAME pMH-1

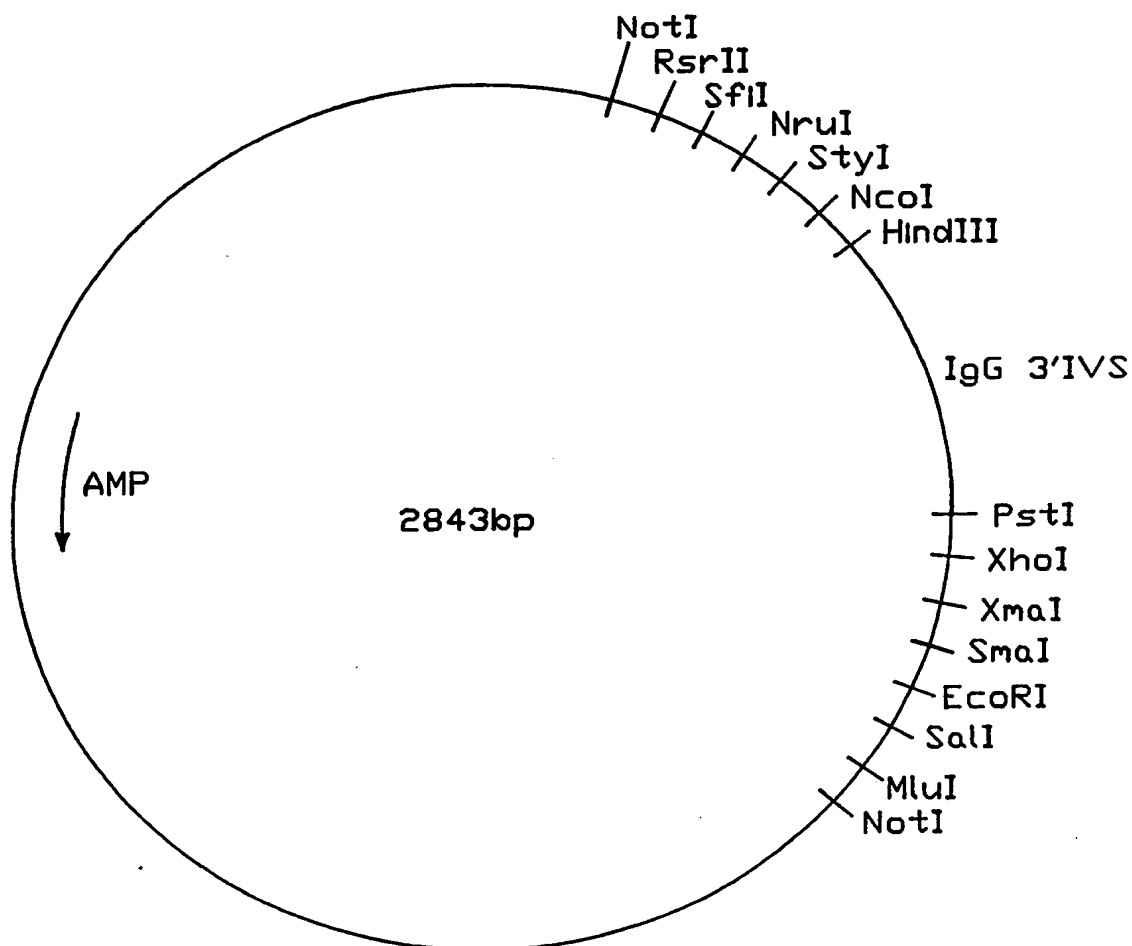
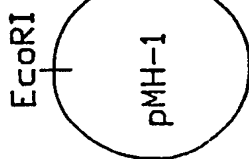
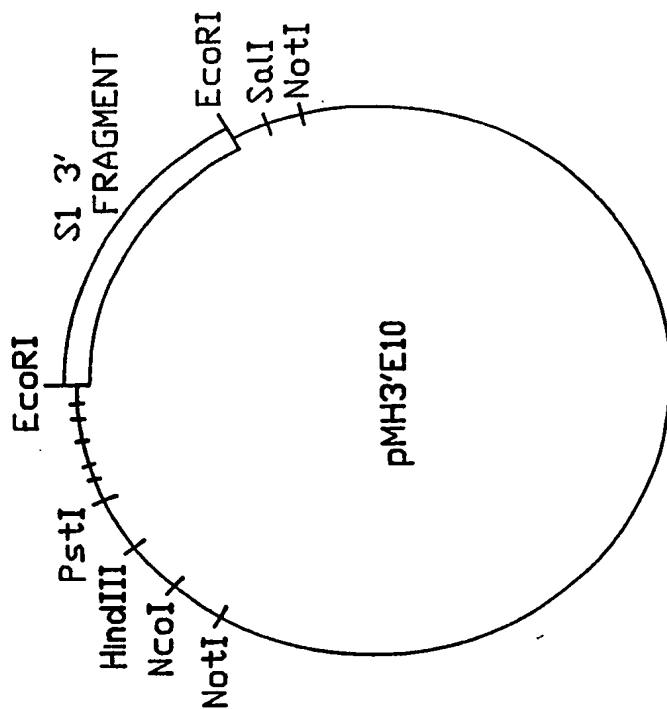


FIG.-6

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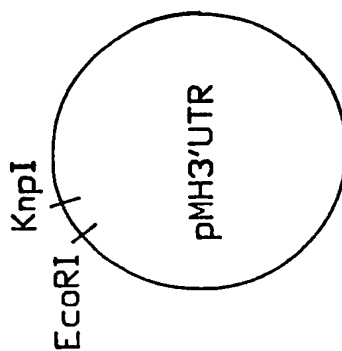
- PLASMID [pMH3'E10] CONTAINS ~ 8.5Kb EcoRI FRAGMENT OF THE 3'-END α S1 CASEIN. pMH-1 WAS CUTW/EcoRI + LIGATED TO THIS FRAGMENT



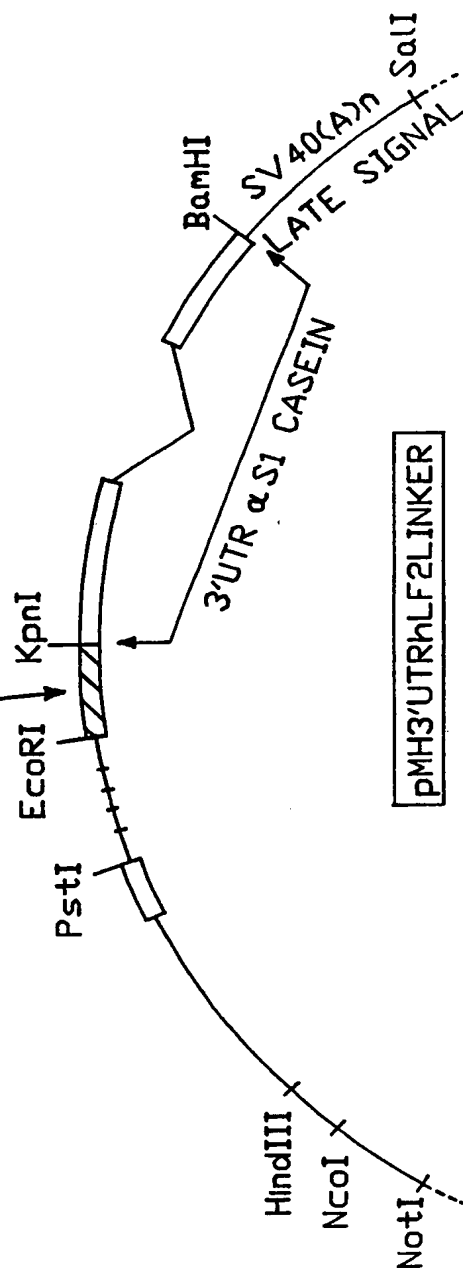
8.5 Kb α S1 CASEIN
+ 3' EcoRI FRAGMENT

EcoRI
DIGEST

- PLASMID [pMH3'UTRhLF2LINKER]



(FRAGMENT 7)
3' TERMINAL
+ hLF CODING
SEQUENCE
(~ 20bp)
CUT W/EcoRI + hLF CODING
AND KpnI



[pMH3'UTRhLF2LINKER]

FIG.-7A

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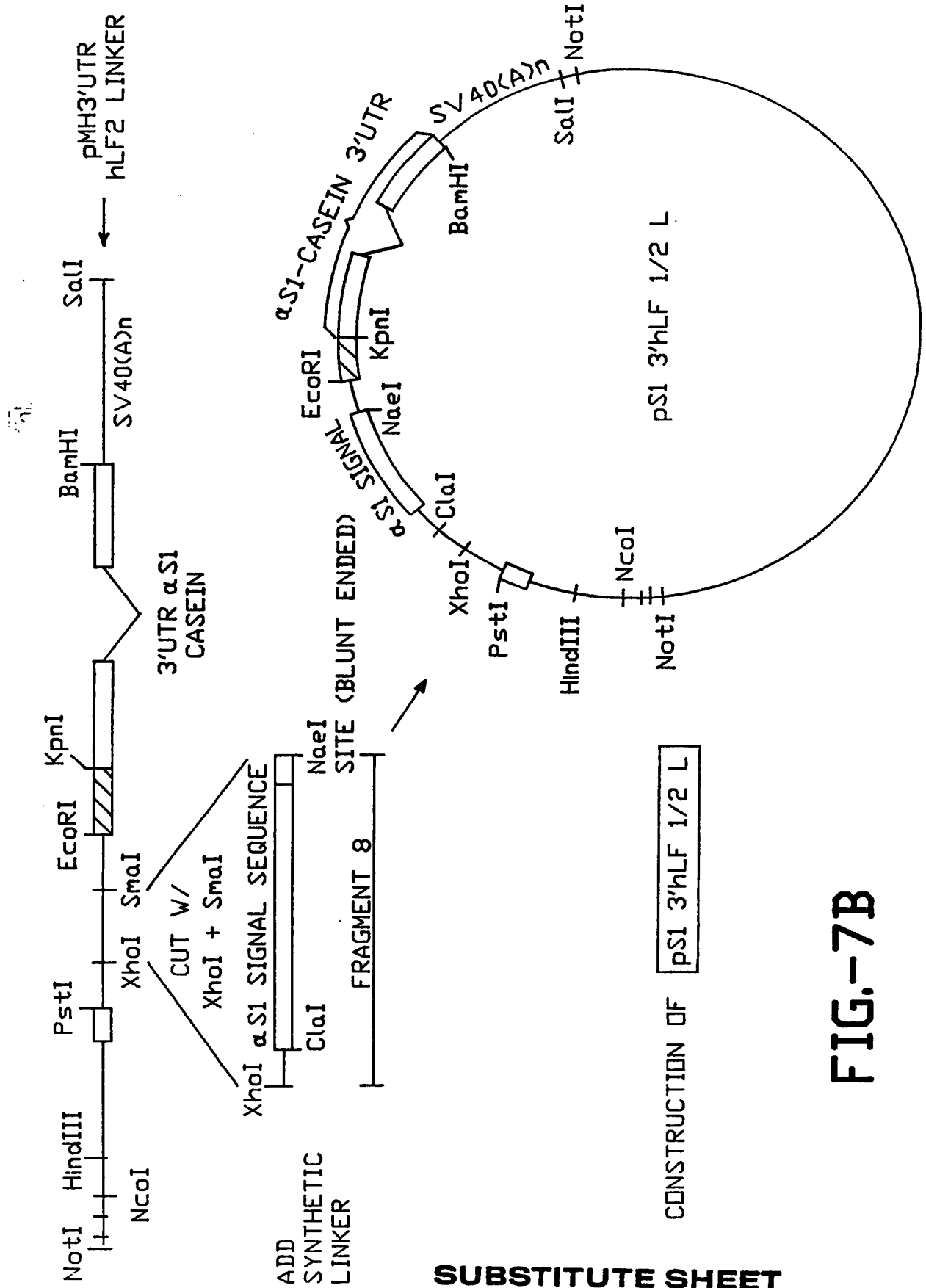
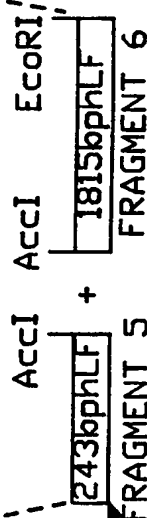


FIG.-7B

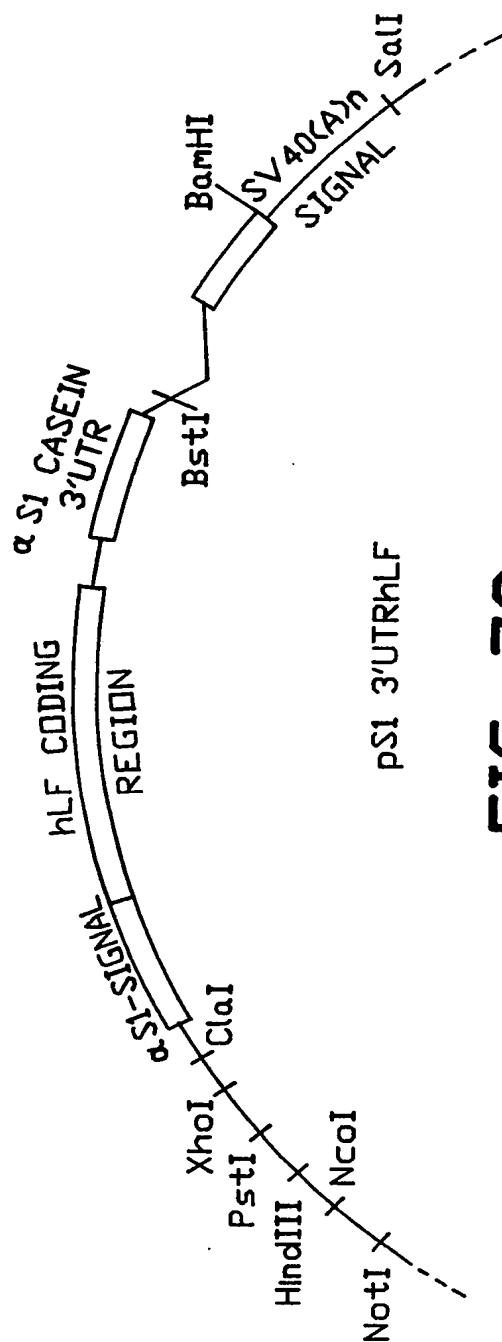
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CONSTRUCTION OF [p1 3'UTRhLF]

pS13'hLF 1/2 L (SEE p.5) CUT W/ NaeI AND EcoRI:



ADD: 2hLF
FRAGMENTS
5 AND 6



pS1 3'UTRhLF

FIG.-7C

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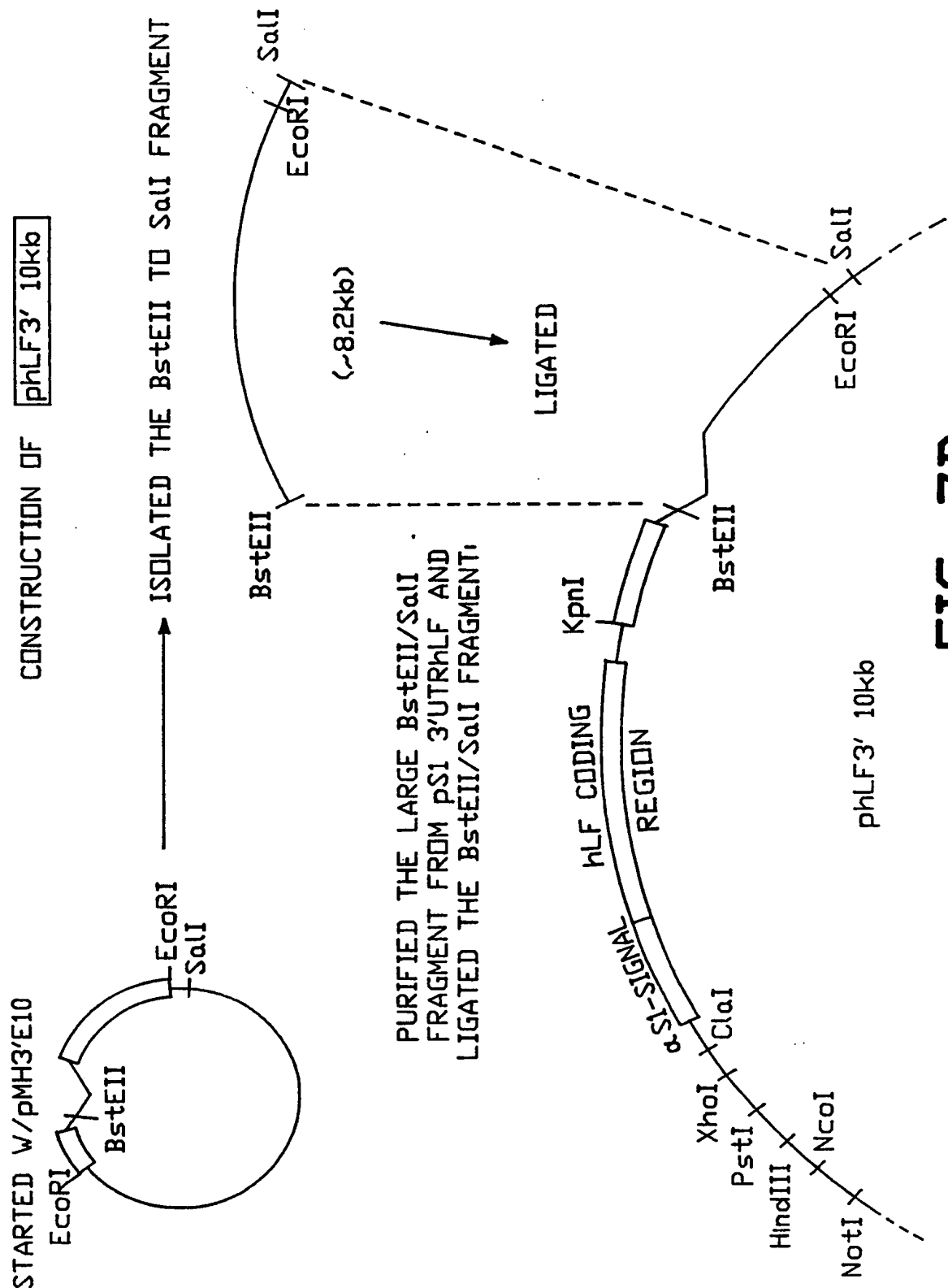
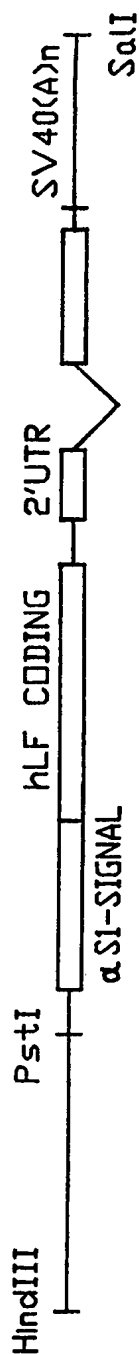


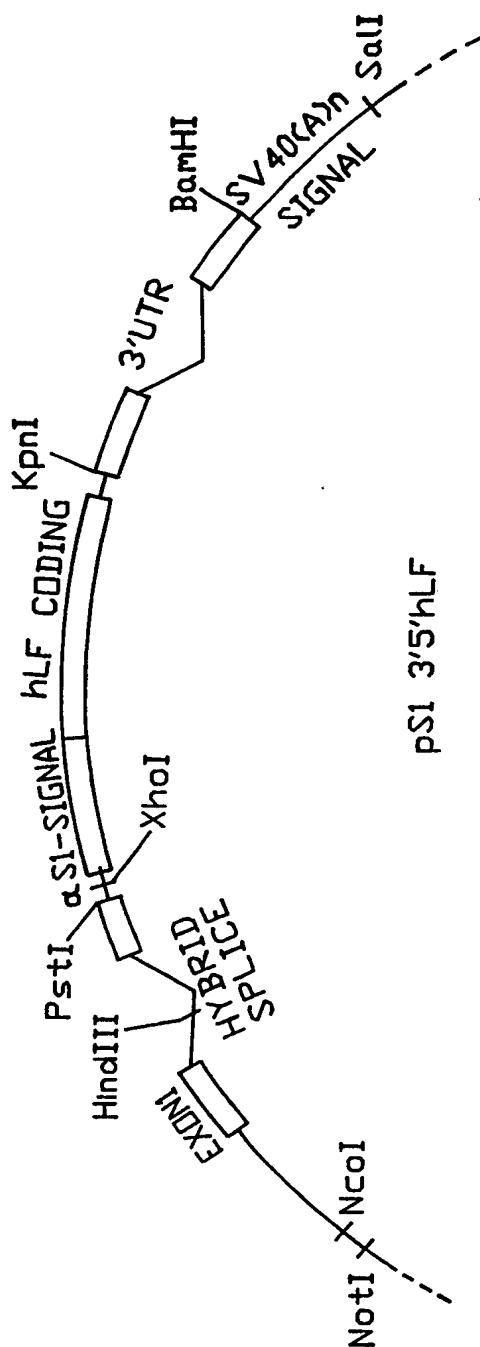
FIG.-7D

CONSTRUCTION OF **pS1 3'5'hLF**

STARTED W/PS1 3'UTRHLF. CUT W/HindIII AND SalI AND PURIFIED THIS FRAGMENT CONTAINING THE α S1-CASEIN SIGNAL SEQUENCE, hLF CODING REGION, α S1UTR AND SV40(A)_n.



SUBCLONED INTO HindIII/SalI
CUT pMHS15' FLANK TO YIELD:

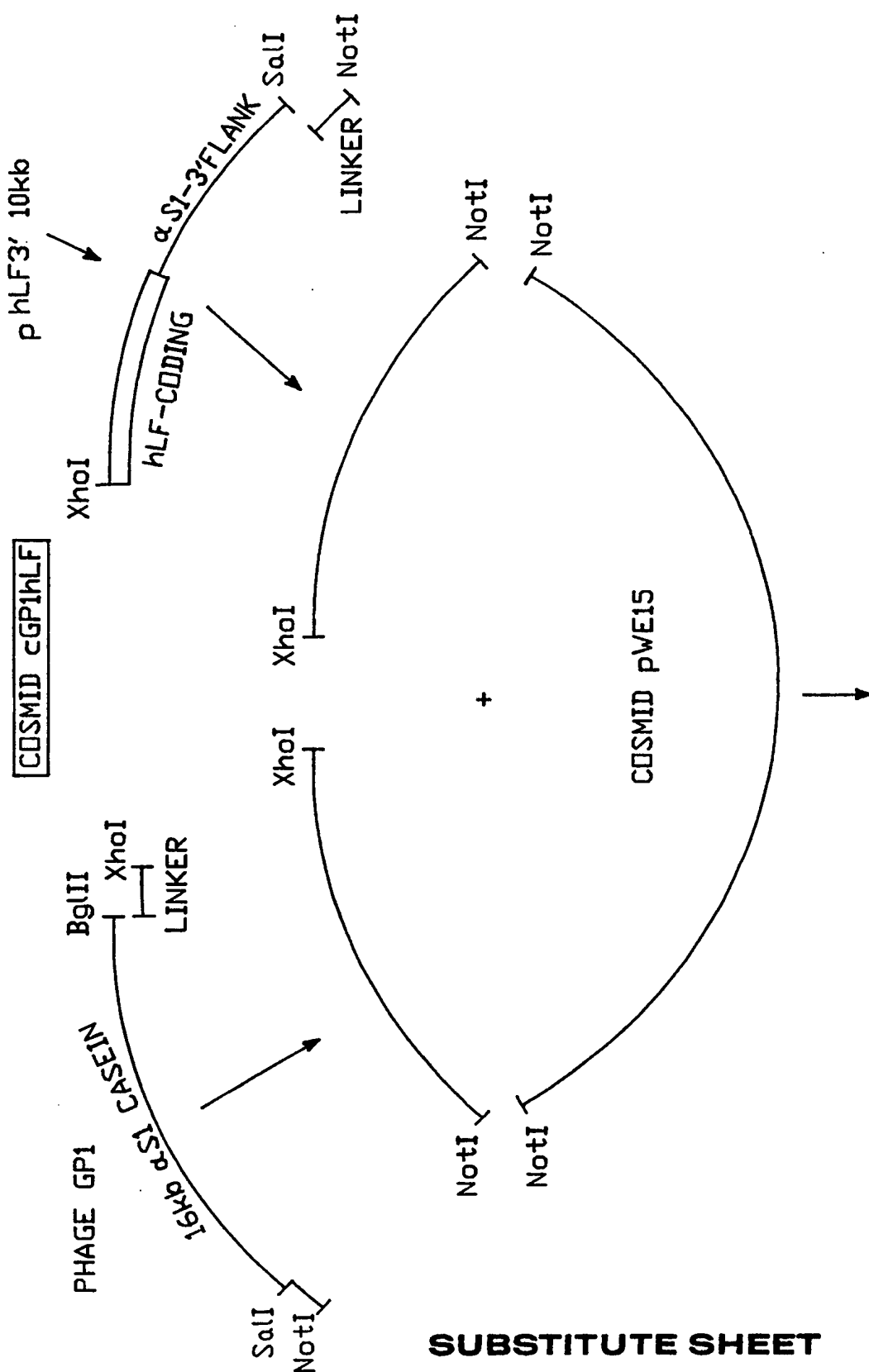


pS1 3'5'hLF

NOTE: **pS1 5'hLF** WAS MADE BY CUTTING p3'5'hLF W/KpnI AND BamHI, FOLLOWED BY BLUNTING THE ENDS AND RELIGATING. THIS ELIMINATES THE SPLICED 3'UTR REGION.

FIG.-7E

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3 WAY LIGATION. THE DNA FROM THIS COSMID IS PREPARED BY CUTTING WITH *NotI* AND PURIFYING THE EXPRESSION SEQUENCE PRIOR TO MICROINJECTION.

FIG.-7F

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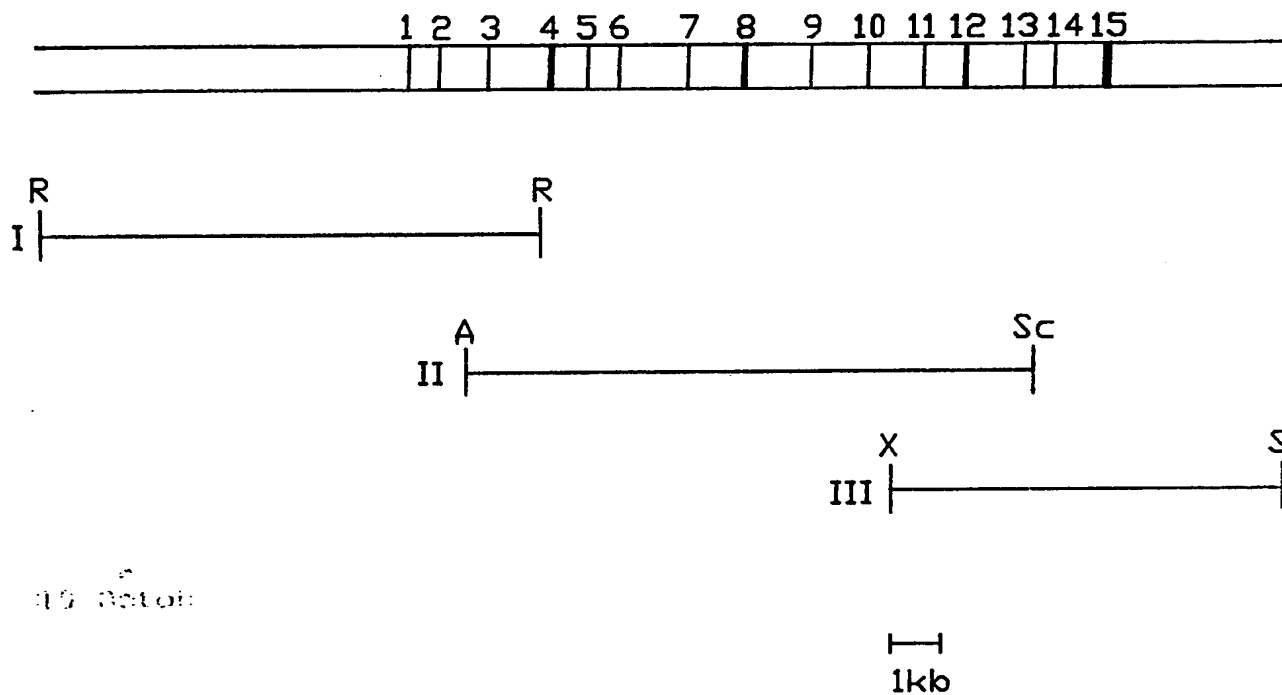


FIG.-8A

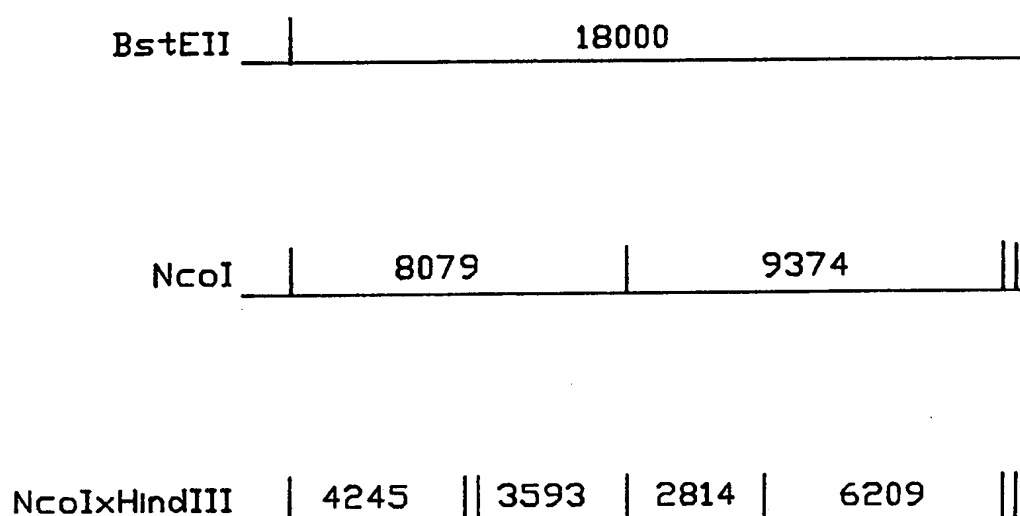
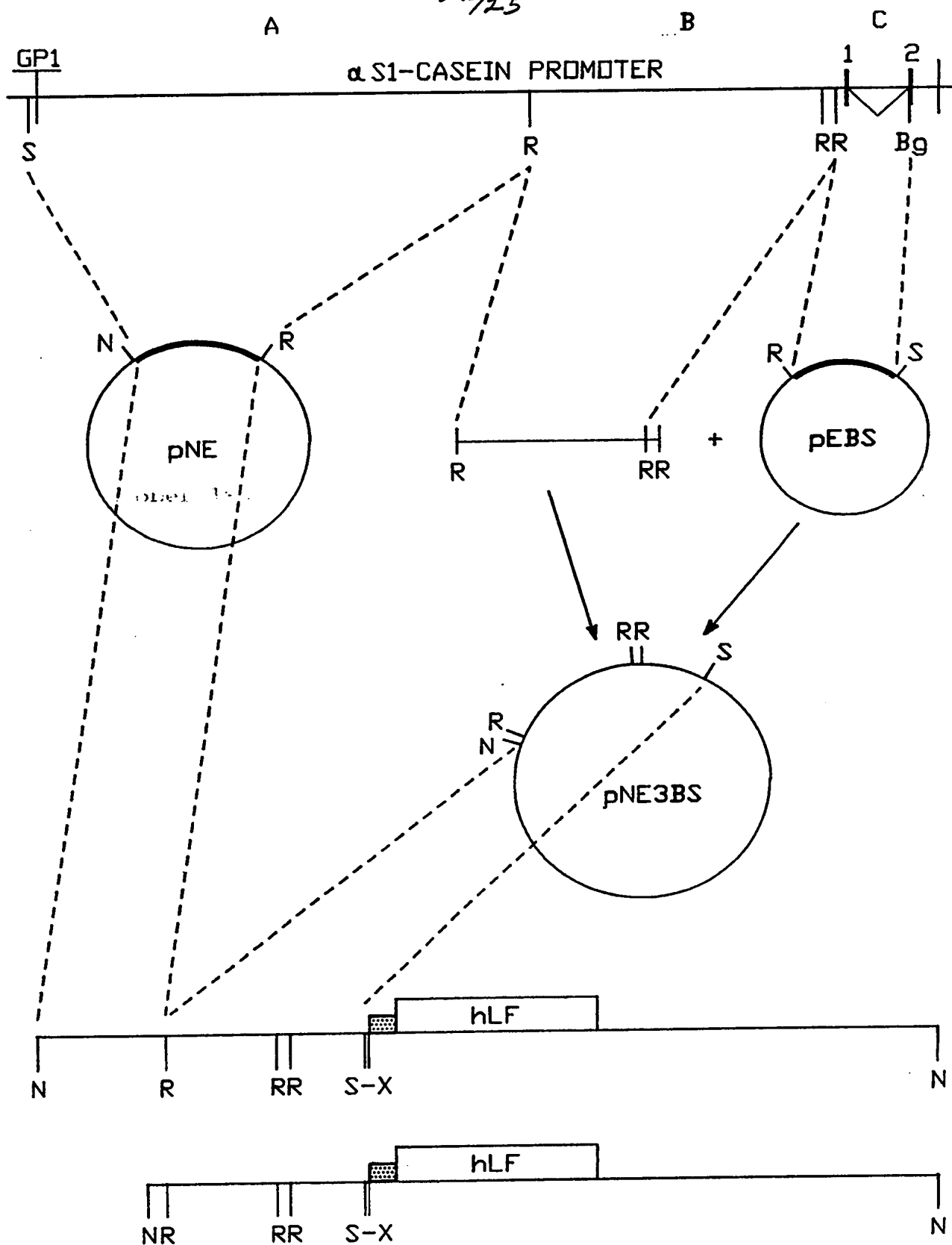


FIG.-8B

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S=SalI R=EcoRI Bg=BglII N=NotI X=XhoI

FIG.-9 SUBSTITUTE SHEET

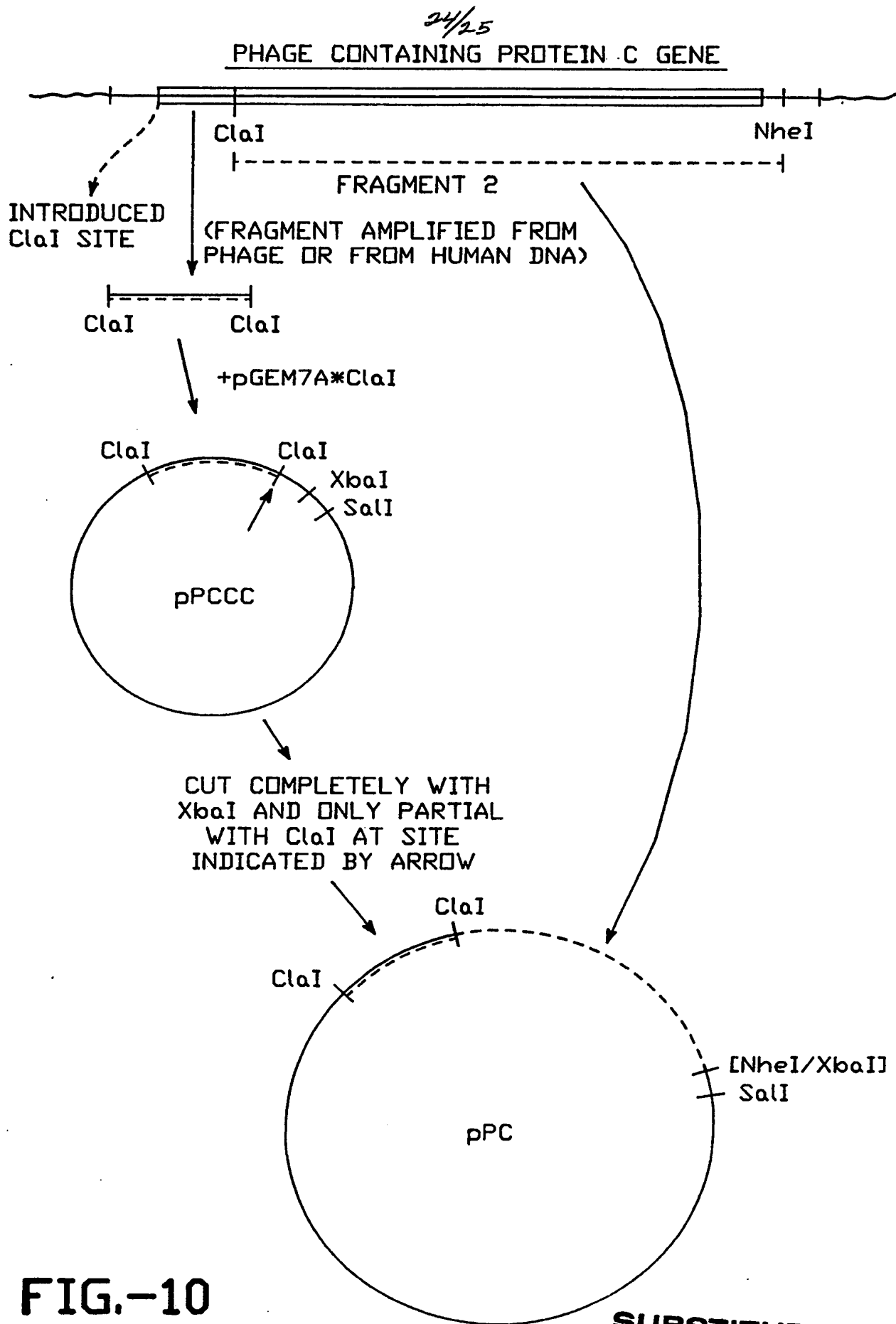


FIG.-10

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Shown below is the DNA sequence of the hybrid splice. The predicted intervening sequence (shown in lower case) consists of the 5'-end of IVS-1 from bovine α S1 casein (from position +54 to +180 with respect to the start of transcription) fused to the 3'-end of a human IgG splice sequence. The HindIII site (in bold type and underlined) derives from the IgG sequence and marks the junction between the α S1 and IgG splice sequences. The 5'-end upper case sequence depicts the complete exon one of the bovine α S1 casein gene. The 3'-end upper case sequence represents the splice junction of the IgG gene through to the PstI site (CTGCAG) incorporated in the cloning vector, pMH1.

```

5'- ATCACCTTGA TCATCAACCC AGCTTGCTGC TTCTTCCCAG
    TCTTGGGTTA AAG gtattatgta tacatataac aaaatttcta tgattttcct ctgtctcatc
    tticattctt cactaatagc cagttgtaac ttictatgt gattgcaagt attggtactt tcctatgata
    tactgttagc aagcttgagg tgtggcaggc ttgagatctg gccatacact tgagtgaaca tgacatccac
    ttgcctttc tctcccacag GTGTCCACTC CCAGGTCCAA CTGCAG -3'
  
```

FIG.-11

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US90/06874**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(5):C07H 21/00; A23C 9/00; C12N 15/00
U.S.CL.: 536/27; 426/580; 800/2

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	426/580; 435/172.3, 317.1; 536/27; 800/2, DIG 1

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

Databases: DIALOG (Files 55, 311, 312, 154), USPTO Automated Patent System (File USPAT, 1975-1991). See attachment for search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	US, A, 4,873,316 (MEADE ET AL.) 10 October 1989, see the entire document.	1-62
Y	Bio/Technology, Volume 7, Issued May 1989, Clark et al., "Expression of human anti-hemophilic factor IX in the milk of transgenic sheep", pages 487-492, see the entire document.	1-62
Y	Bio/Technology, Volume 6, No. 10, Issued October 1988, Van Brunt, "Molecular Farming: transgenic animals as bioreactors", pages 1149-1154, see the left column on page 1154 in particular.	29-53
Y	Bio/Technology, Volume 6, Issued February 1988, Simons et al., "Gene transfer into sheep", pages 179-183, see the entire document.	1-62

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

26 February 1991

15 MAR 1991

International Searching Authority

Signature of Authorized Officer

ISA/US

Jasemine C. Chambers
Jasemine C. Chambers

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y,P	Bio/Technology, Volume 8, Issued February 1990, Bühler et al., "Rabbit β -casein promoter directs secretion of human interleukin-2 into the milk of transgenic rabbits", pages 140-143, see the entire document.	1-62
Y	Bio/Technology, Volume 5, Issued November 1987, Gordon et al., "Production of human tissue plasminogen activator in transgenic mouse milk", pages 1183-1187, see the entire document.	1-62
Y	Theriogenology, Volume 29, No. 1, Issued January 1988, Riery et al., "Gene transfer by pronuclear injection in the bovine", page 224, see the entire abstract.	1-40, 54
Y	Theriogenology, Volume 25, No. 1, Issued January 1986, Loskutoff et al., "Gene microinjection in bovine embryos facilitated by centrifugation", page 168, see the entire abstract.	1-40, 54
Y,P	The New York Times National, Issued 08 June 1990, Schneider, "Texas researchers develop 4 gene-altered calves", see the entire article.	29-40
Y	Proceedings of the National Academy of Sciences, Volume 83, Issued July 1986, Johnson et al., "Replication of a plasmid bearing a human Alu-family repeat in monkey COS-7 cells", pages 4660-4664, see the entire document.	55-62
Y	Journal of Dairy Science, Volume 72, Issued October 1989, Bremel et al., "Alteration of Milk Composition Using Molecular Genetics", pages 2826-2833, see the entire document.	41-53

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Biochemical and Biophysical Research Communications, Volume 135, No. 3, Issued 28 March 1986, Lee et al., "Methylation analysis of a plasmid containing a mammalian cell cycle regulatory sequence after transient transfection into the host cell", pages 942-949, see the entire document.	55-62
Y	WO, A, 88/01648 (HOPP) 10 March 1988, see the entire document.	1-62
Y,P	WO, A, 90/05188 (ARCHIBALD ET AL.) 17 May 1990, see the entire document.	1-62

Attachment to PCT/ISA/210, Part II.

II. Fields Searched Search terms:

transgenic, bovine, cow, milk, lactoferrin, recombinant,
inventors' names, DPN, methylate, replicate, integrate, review.

PCT/US90/06874

Attachment to Form PCT/ISA/210

Part VI. Observations where unity of invention is lacking

I. Claims 1-28, drawn to a transgene, classified in Class 536, subclasses 27, for example.

II. Claims 29-40 and 54, drawn to a transgenic bovine species and a method of making the same, classified in Classes 800, subclass 2, for example.

III. Claims 41-53, drawn to milk from a transgenic bovine species, classified in Class 426, subclass 580, for example.

IV. Claims 55-62, drawn to a method of producing a transgenic non-human mammal having a desired phenotype, classified in Class 800, subclass 2, for example.

GENERATION OF TRANSGENIC DAIRY CATTLE USING 'IN VITRO' EMBRYO PRODUCTION

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We have combined gene transfer, by microinjection, with 'in vitro' embryo production technology, enabling us to carry out non-surgical transfer, to recipient cows, of microinjected embryos that have been cultured from immature oocytes. Using this approach, we have established 21 pregnancies from which 19 calves were born. Southern blot analysis proved that in two cases the microinjected DNA had been integrated in the host genome.

Heterologous protein production in the mammary gland of dairy animals may become an important alternative to cell-culture based expression systems. The feasibility of this technology in the mouse system has been demonstrated in several reports in which high level, tissue-specific expression of foreign proteins in milk was obtained¹⁻³. Several groups have reported limited success of germline transformation by applying essentially the same approach to other species, in particular animals with a relatively short generation time such as rabbits, sheep and pigs⁶⁻¹¹. In these experiments, the supply of fertilized oocytes was, like that in the murine system, based on superovulation followed by 'in vivo' fertilization. After surgical removal of the zygotes followed by microinjection, embryos were allowed to develop in the oviduct of recipients into which they were placed via another surgical procedure.

Dairy cows would seem to be the optimal species for production of very large quantities of heterologous protein in the mammary gland since they can produce over 10,000 liters of milk per year that contains 35 grams of protein per liter¹². However, the generation interval of cattle is about 2 years and cows normally produce only one offspring per gestation. In addition, the logistics of supplying the large numbers of bovine zygotes that are required for the production of transgenic animals from live animals using conventional procedures is cumbersome. Moreover, superovulation and artificial insemination followed by flushing of oviducts of donor cows and oviductal transfer to recipients is very costly because of the

two surgery steps involved.

Here we report the generation of transgenic dairy cattle based on a novel approach in which gene transfer is combined with an 'in vitro' embryo production procedure, thus enabling non-surgical transfer of microinjected embryos that have developed from immature oocytes. Using this approach we demonstrate, for 2 cases, the successful incorporation of microinjected DNA in the genome. The integrated DNA construction contains signals for directing expression of the human iron binding protein, lactoferrin, to the mammary gland.

RESULTS

Oocyte maturation and fertilization. Bovine oocytes were collected by aspiration of follicles present on ovaries obtained from local slaughterhouses. For this study a total of about 2500 oocytes were used. On average we performed two aspiration sessions per week. The yield of aspirated oocytes was highly variable from day to day, with a mean daily number of about 150. Maturation and fertilization were analyzed by cytological analysis. Maturation was defined as the breakdown of the nuclear membrane, the appearance of the first polar body and a metaphase plate. For fertilization, frozen semen from three different bulls was used, each with excellent characteristics with respect to genetic background, field perfor-

TABLE 1 Efficiencies of the steps involved in the process from immature bovine oocytes to transgenic calves.

Step	Total No.	Percent*
oocytes	2470	—
matured	2297	93
fertilized	1358	61
injected	1154	85
survival	981	85
cleavage	687	70
transferred	129 [‡]	19
pregnant	21	21
integration	2	10

*Percentages indicate the proportion of embryos or cells that successfully complete each step.

[‡]Sixty-nine transfers of single blastocytes resulting in 7 pregnancies; 30 transfers of twinned embryos, resulting in 14 pregnancies.

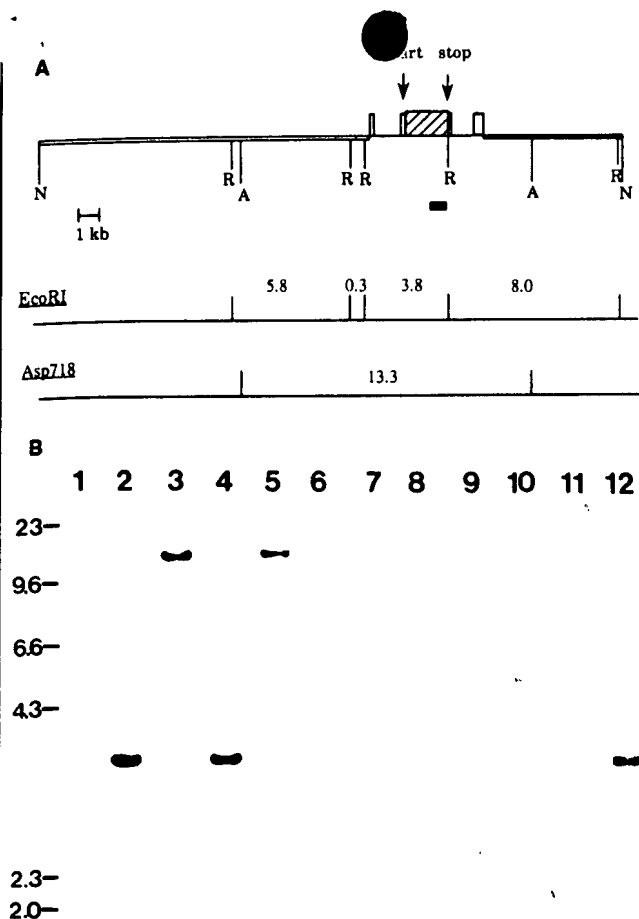


FIGURE 1 (A) Structure of the bovine casein-hLF transgene. The coding sequence of the hLF cDNA is depicted by a hatched box. The position of the translational start and stop codon is indicated. The 5' and 3' untranslated regions are encoded by α S1-casein exons (open boxes). Intervening sequences interrupting these exons are represented by a single line. The expression unit is surrounded by flanking sequences derived from the bovine α S1-casein gene (indicated by a double line). Positions of restriction enzyme sites are indicated by the following symbols: R, EcoRI; A, Asp718; N, NotI; The NotI sites are not present at the indicated positions in the bovine α S1-casein gene itself, but were introduced by synthetic linkers. The black bar represents the position of the probe used to detect the presence of the transgene. Sizes of the fragments (in kbp) obtained after digestion with EcoRI or Asp718 are shown at the bottom. (B) Southern blot analysis of DNA extracted from various tissues. Ten μ g of DNA was loaded per lane. Fragment size markers in kbp (HindIII digest of lambda DNA) are indicated on the left. Lane 1, EcoRI digested human DNA (isolated from blood); lane 2, EcoRI digested DNA from calf #4 isolated from blood; lane 3, Asp718 digested DNA from calf #4 isolated from blood; lane 4, EcoRI digested placental DNA from calf #4; lane 5, Asp718 digested placental DNA from calf #4; lane 6, EcoRI digested DNA from calf #15 isolated from blood; lane 7, Asp718 digested DNA from calf #15 isolated from blood; lane 8, EcoRI digested DNA from calf #15 isolated from ear tissue; lane 9, Asp718 digested DNA from calf #15 isolated from ear tissue; lane 10, EcoRI digested placental DNA from calf #15; lane 11, Asp718 digested placental DNA from calf #15; lane 12, EcoRI digested DNA isolated from the tail of a transgenic mouse harboring the same construct.

mance and ease of calving. For each batch of semen the 'in vitro' fertilization conditions (heparin concentration and sperm number) were optimized to obtain normal fertilization rates ranging from 50 to 70% as determined by the presence of two pronuclei and a sperm tail. We used either one of two techniques for selection of motile sperm: the swim-up technique¹³ and centrifugation through a Percoll gradient (J. Parrish, personal communication). No

significant differences in fertilization rates between these methods were recorded. The efficiencies of these and the following steps are shown in Table 1.

Pronuclear injection. In order to visualize pronuclei, fertilized oocytes were centrifuged at $14,500 \times g$ for 8 minutes¹⁴. The time window in which pronuclei could be visualized appeared to be smaller than the period in which murine pronuclei are visible. Cytologically, i.e. after fixation and staining with aceto-orcein, pronuclei are clearly detectable from 16 hours up to 23 hours after fertilization, whereas using interference contrast optics pronuclei can be visualized after centrifugation between 18 hours and 23 hours after fertilization. The number of oocytes in which pronuclei could be visualized during this period was about 10% smaller than the number that was expected based on the cytological data. Possibly, some nuclei are hidden behind the yolk cap that is formed after centrifugation. Pronuclear injection was performed essentially as described¹⁵. About 10% of the zygotes collapsed after microinjection and were discarded.

Embryo development. After microinjection, the embryos were transferred to microdrops of medium conditioned by bovine oviductal epithelial cells as described¹⁶. Embryo development was evaluated 9 days after the start of maturation. The development rate varied by about 5% depending on the sperm donor used. In addition, microinjected embryos developed, in general, slower than control embryos that had been centrifuged but not microinjected. The formation of the blastocyst cavity was delayed by approximately 1 day. Also, fewer microinjected embryos developed to the morula/blastocyst stage than control embryos (19% vs. 20–25%, Table 1).

Embryo transfer. The synchronization schedule was set up so that recipients started estrous on the same day at which oocytes were aspirated from slaughterhouse ovaries (i.e. start of maturation is day 1). Recipients received 9-days old embryos, at which time they have developed to the compact morula or early blastocyst stage. These embryos are one day ahead in development compared to the stage of the estrous cycle of the recipients. In case of two microinjection sessions on subsequent days, one group of recipients was used that were in synchrony with the first batch of oocytes collected. Transfers of embryos that developed from oocytes aspirated on the day of the start of estrous gave better results than embryos from oocytes obtained one day later. Due to the somewhat delayed development of microinjected embryos, there appeared to be a better synchrony between the recipients and the first group of embryos. Recipients received two embryos when the quality grade (according to Linder and Wright¹⁷) was fair to poor and only one single embryo when the quality grade was excellent to good. Each pregnant recipient that received 2 embryos carried only one fetus to term. The overall pregnancy rate was 21%, which is significantly less than the rates reported by others with non-microinjected embryos which had developed 'in vivo'^{18,19}. In the experiments reported here, no transfers with non-injected embryos were performed.

A total of 21 pregnancies were established (confirmed by rectal palpation 45–60 days after transfer). During pregnancy 2 fetuses were lost. One recipient aborted spontaneously for unknown reasons at 7.5 months of gestation. The second fetus, collected at slaughter of the recipient at 3 weeks after the calculated day of parturition, was a full grown dead calf having an abnormal embryonic development called 'schistosoma reflexum'. In both cases no intact DNA could be isolated for analysis. Nineteen calves were born after normal pregnancies. One of these calves died during parturition, and a second, 24 hours after birth, because of pneumonia following accidental

inhalation of milk. A third calf, born after a pregnancy of 10 months and with a body weight of 70kg was euthanized at an age of 3 weeks. Pathological analysis indicated that the animal was suffering from sepsis due to chronic omphalephlebitis. Tissues that could be analyzed from the three dead calves contained no integrated human lactoferrin (hLF) sequences. Therefore, the cause of their death is unlikely to be related to transgene integration. The remaining 16 calves are in excellent health.

Structure of the transgene. The fragment used for injection was designed to express hLF in the mammary gland of a lactating cow. It consists of the coding region of the hLF cDNA²⁰ fused to the bovine α _{S1}-casein signal sequence and flanked by the 5' and 3' untranslated regions of the bovine α _{S1}-casein gene. Both untranslated regions are interrupted by an intervening sequence. These introns were included since several groups have shown that the presence of intervening sequences can dramatically increase expression of cDNA-based constructs both in tissue culture and in transgenic animals^{21,22}.

Expression of the cDNA is controlled by regulatory elements within 15 kbp of 5'-flanking and 6 kbp of 3'-flanking sequences from the α _{S1}-casein gene. These sequences have been shown to contain elements that are responsible for tissue-specific expression of heterologous genes in transgenic mice⁵ (and our own unpublished observations). A schematic drawing of the casein-hLF transgene is shown in Figure 1.

DNA analysis. DNA was isolated from placenta, blood and ear tissue from all calves. Southern blot analysis using hLF cDNA as a probe indicated that in tissues of two calves (#4 and #15) transgene sequences had been integrated into the host genome. Calf #15 (a female) was mosaic for integration of the transgene: placental tissue was positive, whereas in blood and ear tissue no hLF sequences could be detected. The copy number in the placenta was 1-2. The restriction enzyme map of the transgene was different from that expected based on the map of the casein-hLF plasmid (Fig. 1) and based on the pattern obtained in many individual transgenic mice (data not shown). Apparently, a rearrangement had occurred involving a deletion of part of the DNA construct. It is not clear whether this rearrangement event is related to the fact that the transgene could not be detected in all tissues. In mice it has been shown that over 30% of all transgenic animals born are mosaic²³.

Calf #4 (a male) showed, in all three tissues, the same hybridization pattern that was identical to the expected one. Restriction digestions with different enzymes indicated that head-to-tail concatemers of intact copies had integrated and there was no indication of rearrangements. Copy numbers were estimated by comparing the intensities of the transgenic band with bands resulting from hybridization of the hLF probe to human DNA (Fig. 1). In calf #4 between 5 and 10 copies of the transgene had integrated in all three tissues examined.

DISCUSSION

This work proves the technical feasibility of transgenesis in the bovine system: in 2 out of 19 calves born from microinjected zygotes, the introduced DNA was integrated into the host genome. In parallel experiments, the transgenesis rate in mice that received the same casein-hLF construct was about 10% (data not shown). Thus, based on the limited number of animals born, we tentatively conclude that the transgenesis rates in cattle and mice are, in our hands, the same.

Most attempts to produce transgenic cattle have relied partly or totally on *in vivo* procedures^{24,25}. Fertilized

oocytes were retrieved from superovulated and artificially inseminated cows. Microinjected zygotes were transferred by surgery either directly into the oviduct of recipient cows or into temporary hosts like sheep or rabbits. Obviously, the '*in vivo*' production of zygotes is much more labor intensive than the '*in vitro*' production. Also a larger number of additional animals and veterinary surgeons are involved. Another major disadvantage of the '*in vivo*' procedure is that the stage of pronuclear development at which the zygotes are isolated and processed varies considerably²⁶, and therefore the most suitable time for microinjections cannot be determined. In addition, up to 50% of the embryos transferred into ligated oviducts in living animals cannot be recovered²⁷. Finally, the development of the embryos cannot be followed in the oviduct of temporary hosts, and therefore the stage at which the embryos are recovered is unpredictable. Some of the disadvantages of the '*in vivo*' procedure may be compensated to some extent by the fact that embryos produced by this method have, in general, a better developmental potential²⁸.

All the disadvantages described above are circumvented when using the '*in vitro*' procedures described here. A large number of aspirated oocytes are matured and fertilized simultaneously and their pronuclei can be injected within a short time. Development of the individual embryos can be monitored closely, which makes it possible to transfer embryos at very specific stages of development. The '*in vitro*' culture of embryos up to the morula/blastocyst stage also allows for additional manipulations such as biopsy of blastomers for transgenesis detection, sexing and cloning. It is expected that the incorporation of these, in part established, technologies in our '*in vitro*' program will further reduce the number of recipients needed to generate (larger numbers of) transgenic cattle.

The procedures described in this paper make use of oocytes obtained from ovaries of slaughtered cows. As a consequence, the genetic background of these animals is unknown. Recent developments in the efficient retrieval of oocytes from live animals using echoscopy^{29,30} make it possible to generate transgenic calves that have a defined genetic background both from the maternal and the paternal lineage.

EXPERIMENTAL PROTOCOL

Oocyte source. Ovaries were collected at a local abattoir and transported to the laboratory in a insulated container at 30-32°C. Oocytes, together with follicular fluid, were aspirated from 2-8 mm diameter follicles and pooled into 50 ml conical tubes. Cumulus-oocyte complexes (COC) were allowed to settle into a pellet, after which the supernatant was discarded and the pellet washed in 50 ml TL-Hepes³⁰. COC, containing several intact, unexpanded cumulus cell layers, were selected and isolated under a dissecting microscope at 15× magnification, washed four times in 10 ml TL-Hepes, once in 2-3 ml TCM199+10% fetal calf serum (M199)³⁰ and then transferred to 100 µl droplets of M199 medium under paraffin oil (20 COC/droplet). COC were incubated for 23 h in a humidified atmosphere of 5% CO₂ in air at 39°C.

'In vitro' fertilization. Oocytes were fertilized with frozen thawed-sperm obtained from three different bulls in artificial insemination service. Sperm capacitation was facilitated with heparin¹³. Since sperm from individual bulls respond differently to specific fertilization conditions, semen from each lot was tested in advance to determine optimal heparin and sperm concentration required to maximize normal fertilization frequency and to minimize polyspermy. Fertilization conditions for a given bull were selected after screening at heparin concentrations of 0.0, 1.0 and 10.0mg heparin/ml, and at 1.0, 2.0 and 4.0 × 10⁶ motile sperm/ml. Since the proportion of sperm that survives freezing and thawing varies from bull to bull (approximately 30-60% for the bulls used here) sperm preparations were enriched for live, motile sperm by a "swim-up" procedure¹³; alternatively, sperm were centrifuged through a percoll gradient (J. Parrish, personal

communication). After isolation of the motile portion, sperm were counted on a hemocytometer, diluted to an appropriate concentration to yield a 25-fold concentrated stock. The fertilization medium consisted of TALP medium³¹ supplemented with 2.0–10.0 mg/ml heparin (from porcine intestinal mucosa, 177 IU/mg; Sigma)¹³ and if the cumulus was removed prior to fertilization, 1mM hypotaurine, 10mM penicillamine, 20mM epinephrine and 2mM sodium metabisulfite. Matured COC were selected on the basis of expanded cumulus masses for fertilization, washed once in 10 ml fertilization medium, and either added directly to fertilization droplets, or first stripped of their cumulus investment by gentle pipetting through a small-bore, fire-polished pipet and then added to the droplets. Finally, sperm cells were added to a final concentration of 1×10^6 – 2.0×10^6 /ml. After 16–24 h, presumptive zygotes were removed from fertilization droplets. At this point, 20–30 zygotes for each experiment were fixed in 3:1 ethanol:acetic acid for 24 h, stained with 1% aceto-orcein (in 40% acetic acid), and examined to determine fertilization frequency (percentage of sample with 2 pronuclei and a sperm tail). The remaining oocytes were then prepared for microinjection.

Microinjection. The 26 kbp casein-hLF fragment used for microinjection was released by NotI digestion and purified by agarose gel electrophoresis and electroelution. The final DNA concentration was adjusted to 2.5 µg/ml. Batches of 50 cumulus-intact fertilized oocytes were stripped either as described above or by vortexing 2 minutes in 2ml TL-hepes medium in a 10ml conical tube. In order to visualize the pronuclei, cumulus free oocytes were centrifuged in 1ml TL-hepes medium 8 minutes at $14,500 \times g$ in an Eppendorf centrifuge¹⁴. Microinjection was performed essentially as described¹⁵.

Embryo culture. Embryos were cultured from the zygote to the compact morula or blastocyst stage in oviductal-tissue conditioned medium¹⁶. Oviducts were obtained at slaughter and transported at ambient temperature. Luminal tissue from 2–4 oviducts (1–2 cows) was harvested by gently scraping intact oviducts on the outside with a glass slide. The extruded material was washed 5 times in 10 ml TALP-Hepes and diluted in M199 to a tissue:media ratio of 1:50. Media were conditioned in 50 ml "T" flasks containing five ml of oviduct tissue suspension. Conditioned medium was prepared 48 h later from the supernatant after centrifuging tissue suspensions at $13000 \times g$ for 10 min., divided into 1.0 ml aliquots and stored at -20°C until used. Conditioned media frequently contained a proteinaceous precipitate after thawing, which was removed by centrifugation. Droplets were covered with paraffin oil and were incubated for 2 h to permit pH to equilibrate prior to adding zygotes. Zygotes were placed in culture droplets within 2 h after microinjection. Initial cleavage (>2 cells) was assessed 42 h after adding sperm. Media were not changed during the course of incubations. Criteria for normal development consisted of attainment of the compact morula or blastocyst stage.

Embryo transfer. Estrous in recipient cattle was synchronized with a 9-day Norgestamet (Intervet, Boxmeer, The Netherlands) treatment (administered in an ear implant according to the manufacturer), and a 500 µg dose of cloprostanol given on day 7 of the Norgestamet treatment. Estrous occurred within 2–3 days after implant removal. Embryos were transferred non-surgically to recipient heifers 5–7 days after estrous (1–2 embryos/uterine horn). Pregnancy was determined by rectal palpation at 45 to 60 days of gestation.

DNA analysis. DNA extraction, Southern blot analysis and hybridization were performed according to standard procedures³². The probe used in the Southern blotting experiment was a 758 bp EcoRV-EcoRI fragment covering the 3' part of the hLF cDNA²⁰.

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Production of biomedical proteins in the milk of transgenic dairy cows: the state of the art

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We are applying transgenic technology to dairy cattle aiming at the production of biomedical proteins in their milk. Among a number of beneficial points of the transgenic approach, over already existing protein production methods, is the possibility of being able to provide low-volume, high-cost biomedical proteins for intravenous use at low cost as well as high-volume, low-cost biomedical or nutritional proteins at low cost; in a self-replicating and self-duplicating production system and in an environment-friendly way. The potential availability of any such protein, both naturally existing ones or artificially designed proteins, in abundance in milk as a medium, may also be of inspiration to those researchers that are developing controlled drug release methods; drugs delivered by oral route involving proteins or peptides including for those cases where methods for delivery may have been worked out but where the very large quantities of the proteins or peptides involved would make any clinically relevant application prohibitively expensive to the patients and their insurers.

Key words: Biomedical and nutritional protein; Biomedical peptide; Oral administration; Production in milk; Transgenic dairy cow

Introduction

Up to date, several proteins of biomedical importance have been synthesized via recombinant DNA methods; bacterial, yeast, fungal and mammalian cell cultures have been used for this purpose. As a general rule, complex proteins requiring specific posttranslational modifications (such as tissue-type plasminogen activator, erythropoietin and factor VIII) have been produced in mammalian cell cultures, whereas bacterial and yeast fermentation systems have been utilized for simpler proteins (such as insulin, growth hormones and cytokines). There is, how-

ever, great difference in efficiencies, yields and costs in protein production between the two; bacterial and yeast systems being far more superior to those of mammalian cell culture systems. In addition, production in mammalian cell culture systems requires high cost serum, equipment, specialized labour force and an elaborate and expensive QA/QC system.

The choice of the most suitable expression system depends primarily on the protein needed to be produced. For example, oral treatments with specific biomedical proteins and proteins with both biomedical and nutritional properties (sometimes referred to as 'nutraceutical proteins') will require administration of sometimes very large quantities of a specific protein regard-

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less its molecular complexity. In such cases, production via the milk of transgenic dairy cows is of particular suitability as will be demonstrated in this paper. In terms of costs alone, bacterial or yeast systems may be chosen. However, bacterial systems are unsuitable for the production of proteins with a nutritional function with the exception of microorganisms with GRAS status. To resolve this issue, i.e., production of high-volume low-price proteins, especially those needed for oral administration, we have developed a novel protein production method based on transgenic technologies, i.e., the utilization of the mammary gland of dairy cows as the molecular expression system for the category of proteins mentioned above. In this communication, we will discuss the advantages and disadvantages of this production system in comparison with production via other transgenic mammals as well as the current state of the art of the technology. We believe that this technology may, in the future, be also complementary to scientific endeavours aimed at achieving improved methods for delivery of peptide drugs via the gastro-intestinal tract, i.e., production of such peptides or proteins via the milk may be a good starting point for such delivery methods.

The mammary gland as expression system

It has been amply demonstrated that heterologous proteins can be produced in the mammary gland of various mammals (Table 3). The consideration to utilize the mammary gland of dairy animals for large scale production of specific proteins, which need to be administered orally, stems from the fact that the mammary gland of dairy animals is a very cheap protein producer (Table 1). This is due to the large capacity of the protein synthesis and secretory system in the mammary gland of dairy animals and the fact that dairy animals requires low feed and maintenance costs compared to fermentation systems most notably when compared with tissue culture systems. Also, the mammary gland as expression system is by nature very suitable for the production of proteins and peptides that have to func-

tion in the gastro-intestinal tract of humans and which can be administered orally as free proteins or as proteins encapsulated for release in the gut, for example, antibacterial proteins and antibacterial peptides as well as antibodies.

Protein production via mammary epithelial cells has, in general, several distinct advantages over fermentation systems other than cost effectiveness. Since milk can be collected from dairy animals several times a day, the time between actual synthesis of the protein and its harvest can be as short as a few hours, contrasting with fermentation systems. In fermentation systems, the proteins, once they are synthesized and secreted into the medium, are, in most cases, subject to shearing forces, surface denaturation, anti-foam compounds, oxidation and deamidation. These conditions, which can last, in case of mammalian cell culture systems up to several days, i.e., the time between inoculation of the culture and the actual moment of harvest, do damage the proteins in various ways. By contrast, proteins secreted by the mammary epithelial cells enter the protected environment of the milk compartment, i.e., the minuscule space of the alveoli of the mammary gland. The cavity of these alveoli is anaerobic; shear forces are nonexistent; foam is not being formed. In healthy animals, this environment is sterile. Once the actual milking process starts, the alveoli are emptied by contraction of the myoepithelial cells surrounding the alveoli. Here some shearing may take place but this lasts only a few minutes compared to hours or days in case of tissue cultures. Since dairy cows can be milked as frequently as one likes, e.g. four or more times a day, the time between actual synthesis of the biomedical protein and its harvest can be as short as 6 hours or less. Although specific data are not yet available, it may well be that the quality, in terms of microheterogeneity, of proteins thus produced in the mammary gland of transgenic animals is far higher than that produced by any other means as far as the degree of deamidation, oxidation and denaturation is concerned. Also, living animals have a genetic stability that is higher than cell culture-based production systems. In case of liv-

ing animals, the genetic stability is determined by the rate of accumulation of somatic mutations in the stem cells from which the mammary epithelial cells are derived each year in preparation of the lactation period and the rate of accumulation of somatic mutations in the epithelial cells during the lactational period proper. DNA repair systems operate in vivo and aberrant cells may be eliminated through intrinsic immunological surveillance systems. By contrast, mammalian cells growing in tissue culture systems are aneuploid to a large extent and there is no immunological surveillance operating in the fermentor. Consequently, the frequency of amino acid substitutions due to mutations in the gene encoding the protein product may turn out to be much higher than in case of production of such proteins within living animals. Research still needs to be conducted to prove or disprove these hypotheses. The outcome of such studies may considerably affect the way regulatory authorities will judge this novel production method. This concerns, of course, primarily biomedical proteins to be used intravenously and where avoidance of an immunological response against 'damaged' epitopes of the protein is a key concern. In terms of downstream processing, production in milk of transgenic animals has the advantage that most heterologous proteins will end up in the whey fraction after caseins are precipi-

tated by means of a simple acidification step or using chymosin. Such a purification procedure can be easily scaled up, also in developing countries, using simple and scalable techniques; i.e., procedures and equipment common in the dairy industry throughout the world.

Many biomedical and/or nutritional proteins can be considered as candidates for production via the milk gland of dairy animals. High-volume, low-cost proteins are obvious candidates. Lactoferrin, human serum albumin and antibodies of the IgG and IgA type are but a few examples of such proteins. In Table 1, a number of additional candidate proteins are listed along with the quantitative market sizes and the equivalent volumes of human blood plasma that would be required to obtain similar quantities. However, whether the production method of choice for proteins will indeed be the utilization of the mammary gland of a living animal depends on a variety of economical and technical factors. Moreover, the economical considerations will depend on the local (bio)technological infrastructure. Thus, the considerations to be taken into account for selecting between tissue culture based manufacturing processes and transgenesis will be different for a developing country than the considerations to be applied in the developed world. This may be especially true for low volume, high price proteins. As mentioned above,

TABLE 1

Calculation of the number of cows needed to produce the required amount of the indicated biomedical protein and compared to the amount of human blood plasma that needs to be fractionated to obtain the same amount

	Estimated US ^a market per year	If expression level in milk is: g/l	Number of cows needed (@10 000 l milk per year)	Human blood plasma levels	Equivalent volume of human blood plasma to be fractioned ^b
Factor VII	—	—	—	0.5 mg/l	—
Factor VIII	120 g	0.01	1.2	0.1 mg/l	1.2×10^6
Factor IX	—	—	—	5 mg/l	—
Protein C	100 kg	0.1	100	5 mg/l	20×10^6
Fibrinogen	200 kg	1.0	20	4 g/l	0.5×10^6
AT III	800 kg	1.0	80	0.2 g/l	4×10^6
Albumin	100 000 kg	2.0	5000	50 g/l	2×10^6

^aAbout the same for European market and Asian market.

^bBased in 100% recovery.

such proteins are, in developed countries, usually made in factories containing cell culture fermentation systems. These production systems require a large amount of high-technology, highly trained and specialised operators. The establishment of such tissue culture systems in a developing country may be very difficult not only from a technical and economical point of view but also because of limitations in the availability of personnel with the proper skills. Thus developing countries may consider to use transgenic animals as 'bioreactors' since such 'bioreactors' renew and multiply themselves by generating their own progeny. High technology is required only at the very beginning, i.e., at the time of generating the transgenic founder animals. Once the founders are made, the transgene is inherited to the offspring in a simple straightforward mendelian fashion.

In conclusion, for high volume, low cost biomedical proteins, dairy animals are economically attractive in both the developing and the developed countries. The actual production phase of the raw material does not require high technology. In addition, when it concerns proteins that have a nutritional purpose and biomedical proteins that need to be taken orally, these proteins, when produced in milk, require relatively little or no purification; concentration of the whey fraction in combination with procedures common to the dairy industry will suffice. It is also likely that the level of contamination due to microheterogeneity is much less compared to proteins made in the tissue culture systems.

The case of cows

In making the choice which animal to use for production via transgenesis it is important to know how long it takes to produce a large enough quantity of the protein to be used for preclinical and clinical trials. In case of lactoferrin (see below), for example, we believe that such trials will require about 100 kg of the protein. Taking this and the future demand for the protein into consideration, cows will be a highly preferable choice

of animal since the transgenic founders, i.e., the F_0 , can be used as the producers of such material. In case of other animals, although they have a shorter generation time, an F_1 or even an F_2 generation must be bred in order to be able to generate enough lactating females to produce 100 kg material (Table 2). Also, high efficiency of modern dairy farm management, cows are easy to multiply using natural mating procedures that can be complemented with artificial insemination and embryo transfer procedures (all currently existing). As far as the protein production costs are concerned, a single cow can produce more than 10000 liters of milk yearly containing as much as 340 kg proteins (mainly caseins) at costs close to \$0.01 per gram.

Even when other farm animals can be used for the large scale production of proteins, there are many other factors that lead to the final choice of the proper animal to be used. It depends, for example, on the type of protein to be used. Bovine milk is most suitable for the production of proteins to be used in infant formula since cow milk is already used for the manufacturing of infant formula. Not using cows as a production system for such proteins would, for example, not only require regulatory approval and consumer acceptance for the novel protein itself, but also for the different type of milk to be used as starting material. Achieving consumer acceptance of infant formula based on, for example, goat milk or pig milk will be a very difficult matter.

Gene Pharming Europe (GPE)

The development of technology that is needed to be able to generate transgenic dairy animals on a routine basis is too expensive to be borne by academic budgets. In order to pursue the possibility of carrying out the necessary research for the technological development within a commercial setting, the authors formulated, in 1988, the concept for a company while being employed by Leiden University. The establishment the company (GPE) followed in 1989. Subsequently, GPE became part of GenPharm International, together with a sister organization in

TABLE 2

Calculation of the number of lactating animals needed to produce 100 kg of biomedical protein for various mammals assuming an expression level of 1 g/l

	Mouse	Rabbit	Pig	Goat	Sheep	Cow
Liters of milk produced per year	0.02	50	250	400	500	10 000
Number of animals needed for 100 kg protein (1 g/l)	5.8×10^6	2000	400	250	200	10
Time from oocyte injection to first milk	14 weeks	17 weeks	12 months	12-8 months	12-18 months	19-29 months

California; GenPharm, USA. The company, GenPharm International, so far, raised over US\$10 million in venture capital and also obtained a number of government grants.

GPE aims at producing biomedical and nutritional proteins which cannot be produced at all by other means or only at an unacceptably high cost in the milk of transgenic dairy cows. The protein which GPE is currently working on is (human) lactoferrin (LF). LF is a member of a group of proteins that play a role in the general defence system of man and animals. This and other antimicrobial proteins are generally found in secreted fluids (milk, tears, sweat, saliva, vaginal secretions and sperm) and in certain cells of the immune system that are specialised in the killing of invading pathogens (neutrophils). Apart from the bacteriostatic function, lactoferrin also plays a role in the transfer of iron from mother to child via the milk. There is reason to believe that this protein exerts its bacteriostatic action by two different mechanisms, i.e., titration of free iron from the environment, thereby reducing its availability for organisms with a high iron demand such as *E. coli* and secondly by direct interaction of LF with the bacterial (outer) membrane. This interaction causes membrane destabilisation and increases its permeability for other compounds among which lysozyme and several classical antibiotics that normally do not act on gram negatives [1].

Recent data indicate that LF is also able to bind to lipopolysaccharide (endotoxin) component of the outer membrane of gram negative bacteria

[2]. This activity is of particular interest as endotoxin is an important mediator of septic shock caused by systemic infection. Prevention of death of mice caused by intravenous injection of a lethal dose of *E. coli* cells with LF has been demonstrated [3]. Whether this effect in mice is indeed caused by endotoxin neutralisation and whether these results will also apply to other animals and to humans remains to be demonstrated.

These activities of LF and related bacteriostatic and bacteriolytic proteins could be also of interest to developing countries in particular if it appears that, in due time, in a clinical setting, LF and other proteins with antibiotic function are active in combating certain forms of bacterial diarrhoea, a disease that kills world-wide about 5 million children annually. If effective, formulations containing such proteins could, in conjunction with breast feeding or as a supplement in infant formula, be used on a continual basis in order to help preventing the disease. It will also be of importance to examine whether such antibiotic proteins will have a beneficial effect on certain opportunistic gastro-intestinal infections often associated with AIDS, with other immunodeficiency diseases and chemotherapy patients.

The possibility of administering to infants, children and patients, antibiotic proteins that are normally present in mothers milk, would offer a distinctive advantage over usage of classical antibiotics as treatment with those compounds is undesirable for various reasons among which the

TABLE 3

Expression systems used to produce biomedical proteins in milk

Mammary gland specific regulatory sequences used	Gene for expressed biomedical protein	Expression level achieved	Animal used	Institution or company involved
WAP	tPA (cDNA)	0-50 mg/l	goat	Genzyme
WAP	CD4 (cDNA)	1-2 mg/l	mouse	NIH
WAP	Protein C (genomic)	1000 mg/l	mouse	American Red Cross
WAP	Protein C (cDNA)	1000 mg/l	pig	American Red Cross
β LG	FIX (cDNA)	0.02 mg/l	sheep	Pharmaceut. Proteins Ltd.
β LG	AAT (genomic)	35 000 mg/l	sheep	Pharmaceut. Proteins Ltd.
β LG	AAT (gneomic)	50-8000 mg/l	mouse	Pharmaceut. Proteins Ltd.
β LG	human serum albumin	10 000 mg/l	mouse	Rhône Poulenc
β -Casein (rabbit)	IL-2 (cDNA)	0.3 mg/l	rabbit	ETH-Zürich
β -Casein (rat)	FSH (2 cDNAs)	10 mg/l	mouse	Baylor
β -Casein (goat)	tPA (CDNA)	3000 mg/l	goat	Genzyme
β -Casein (goat)	CFTR (cDNA)	1 mg/l	mouse	Genzyme
α S1-Casein (bovine)	Urokinase (genomic)	2000 mg/l	mouse	Biogen
α S1-Casein (bovine)	hLF (cDNA)	2000 mg/l	mouse	Gene Pharming Europe
α S1-Casein (bovine)	hLF genomic	30 000 mg/l	mouse	Gene Pharming Europe
α S1-Casein (bovine)	IGF-1 (cDNA)	10 000 mg/l	rabbit	Munich University

danger of developing resistant pathogenic strains. It is hard to imagine that such problems will arise by consuming a protein that all breast fed infants have, during the entire primate evolution, consumed in large quantities during the first months of their life. Obviously, a lot of studies in the clinic and in the field still need to be done in order to establish the efficacy of these proteins. In order to be able to do such studies, large amounts of such proteins are required. This can, obviously, best be done via the milk of transgenic dairy animals as milk of human donors is relatively scarce and insufficient for carrying out clinical studies on a statistically sound basis. The birth of worlds first transgenic calf, named Herman, harbouring the LF gene containing mammary gland specific regulatory sequences is an important first step towards this goal. The tech-

nology underlying this new development in animal breeding has been described [4] and its potential impact on the industry has been highlighted in [5,6].

At GPE's sister organization, GenPharm USA, Mountain View, California, transgenesis of mice, combined with gene knock-out technology, is used to generate mice that serve to mimic human diseases among which are caused by genetic defects or which are caused by environmental factors in combination with certain genetic predispositions. Mice having such genetic predispositions are routinely made, licensed in and sold at the US branch. In addition, with this same technology, transgenic mice have been generated that lack the ability to produce (murine) antibodies (using gene knock-out technology) and have instead acquired part of the human immu-

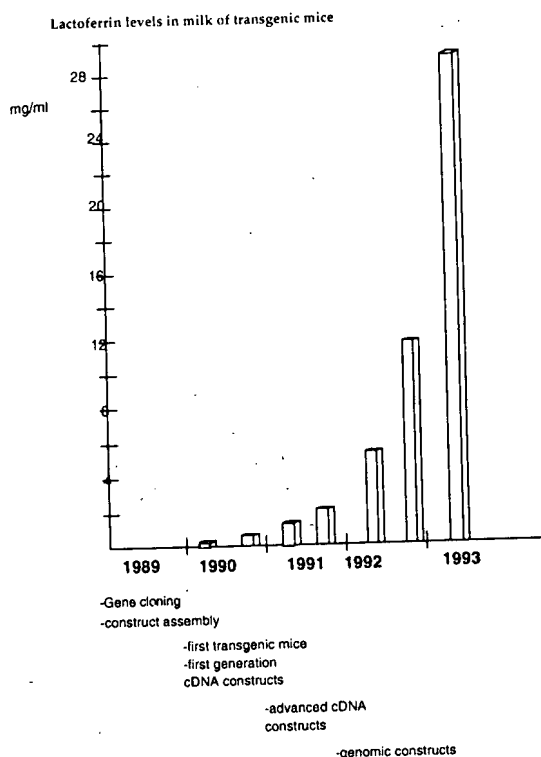


Fig. 1. Lactoferrin expression achieved at GPE in mice over the years using cDNAs and, later on, genomic gene constructs driven by the α S1 casein promoter.

noglobulin locus [7,8]. Upon immunisation with an antigen, including human antigens, these mice respond by generating solely human antibodies; not murine. From such mice, hybridomas will be generated that produce and secrete human antibodies. These antibodies do not need further modification; they do not need to be 'humanised' as is the case for all other hybridoma based antibody production methods. Further information on this subject can be provided by authors on request.

When the first business plan was formulated in 1988, we identified two major technical hurdles of which we were not clear whether they could be overcome. The first one was the uncertainty of whether the biological nature of the bovine embryos would permit integration of a micro injected transgene. This worry, however, was more or less dispelled in 1990 when GPE generated the world's first transgenic bull. The second

issue was whether commercially acceptable levels of heterologous protein expression in the milk of transgenic animals could be obtained. We had set a goal of one g/l as a minimal level (for lactoferrin) when the highest level reported at that time was in the μ g/ml range.

By and large, this problem has also been solved; several proteins have been expressed in the mammary gland of several species including sheep and goats with expression levels that far exceed our expectations of a few years ago. In fact, expression levels of 30 mg/ml for α_1 -antitrypsin in sheep [5] and the same level for LF in GPE's mice are the all time world records for expression levels in any system described so far including microbial and fungal systems; in *E. coli* the maximal expression level ever reported is in the 15 mg/ml range. The expression levels obtained thus far in the mammary gland of the various mammals achieved by various groups, academic or commercial, are shown in the Table 3. In case of LF, the expression levels in our lab have improved from 0.1 to 30 mg/ml over the last 5 years (Fig. 1). The remaining technical issue in this industry is the ease at which transgenic dairy animals can be generated on a routine and predictable basis.

Remaining issues in the transgenic industry

The controversial transgenic pigs harbouring a growth hormone gene or transgenic farm animals with other genes that makes them grow in a manner that affects the health, well being and fertility of the animals, throw a shadow on the technology. From its onset, GPE has set a few simple internal guidelines concerning this new technology. For example, we are only interested in generating animals that produce their new product exclusively in the mammary gland. Whatever is being produced in this gland, it should not affect the health and well-being of the animal. The phenotype of the animal should not become affected by whatever form of genetic modification (a rule that ought to be applied to classical breeding procedures as well). These criteria can easily be explained to the public at large

and will greatly help in achieving acceptance of this technology and its products by the public to the benefit of patients that are in need of such products. As a result of many other forms of public education on this matter, often with the involvement of GPE in general and one of the authors (H.d.B.) in particular, by far the majority (over 70%) of the population is in clearly in favour of this technology where it concerns the production of biomedical proteins and only a few percent is against, as a recent poll has shown.

It would be advisable that other organisations conducting genetic modification of dairy animals elsewhere in the world would adopt similar rules of conduct. This would serve not only to protect the well-being of the animals but also the technology as such and clear the road for applications that certainly will benefit mankind without damaging the reputation of transgenic technology. In addition, having a broad debate among the public and therefore a widely informed public, combined with strong support of the government, as was the case in The Netherlands, is essential to turn this technology into an industry of great significance.

Along with public acceptance, regulatory acceptance of the products produced by transgenic means is also an issue needed to be resolved. For this, it is important that all relevant industries collaborate to achieve this common goal. This is already the case where it concerns developing regulatory guidelines for the forthcoming products. Representatives of all companies involved in transgenic technology are now collaborating with the FDA in order to formulate the contents of a 'Points to Consider' document both for proteins to be used intravenously as for proteins to be used orally (criteria for obtaining a GRASS petition status for nutritional and pharmaceutical proteins). A joint effort of the industries involved in dealing with public issues and with the objective to prevent adverse activities leading to sick animals and animals with poor well-being (see code of conduct section) is highly recommended.

Finally, how successful the industry as a producer of therapeutic proteins will be depends,

among many factors, on the ability of researchers to find ingenious uptake systems of such proteins by cells of the gastro-intestinal tract. This applies also to peptides as peptides too can be easily made via milk. For example, peptides could be made either as a free peptide or as a fusion protein, fused to the hydrophilic tail of kappa-casein via a chymosin sensitive bond. Upon addition of chymosin to milk, the peptide will be released and can be recovered from the whey fraction using simple means (chymosin is the enzyme normally used in the cheese industry in order to precipitate the casein micelles). Alternatively, if peptides have been developed with desirable uptake characteristics in the gut, other peptides can be fused to the first one (at the DNA, i.e., at the transgene level) and expressed in milk in bulk quantities. Also, when the protein or peptide to be taken up by the gut epithelia is hydrophobic or contains one or more transmembrane domains, it can also be expressed in milk. For example, it has been demonstrated that transmembrane proteins, when the expression of their gene is directed to the mammary gland, become an integral part of the apical membrane of the mammary epithelial cells and, as a consequence, become an integral part of the membranes that surround milk fat droplets. Such engineered milkfat membranes may also be interesting vehicles for peptide delivery.

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HEADLINE: GenPharm's bull ready to breed herd of cows to put human milk in a bottle

DATELINE: SAN DIEGO

BODY:

Herman, the first transgenic dairy bull, is ready to start producing a herd of cows that will carry human proteins in their milk, said Jonathan J. MacQuitty, president and CEO of GenPharm International. He expects to have a product on the market by 1995 or 1996.

"His semen has the transgenes and we are starting to breed him now," said MacQuitty at the meeting of the Association of Biotechnology Companies held here last month. A good dairy bull can produce 10,000 calves a year. MacQuitty is confident that the gene will be passed on and that the proteins will be expressed in commercially relevant levels, because his firm has conducted a trial run of its technique in mice.

The genes will help simulate human milk by expressing two essential proteins-human lactoferrin and human lysozyme-that are not present in infant formula. These proteins, which have specific antibacterial and iron-transport properties, were too expensive to produce in adequate quantities by conventional cell culture methods. MacQuitty said that the company has filed several U.S. and foreign patents and has recently received two notices of allowance.

Bristol-Myers Squibb has a collaborative agreement with GenPharm to use the human protein-carrying bovine milk in its infant formula. According to the company, the market for infant formula is more than \$ 4 billion worldwide.

About 75% of babies born each year in the U.S. each year are not breast-fed. Pediatric research indicates that infants fed formula do not do as well as their breast-fed counterparts, said MacQuitty. A study in The Lancet, he said, showed that infants given only formula had more intestinal infections and, in some cases, less-than-optimal mental development.

Transgenic Animal-Based Protein Products Move Toward Clinical Trials

By Gail Dutton

Transgenic animals have been available since 1980, but companies are only now prepared to advance to clinical trials (xenografts from transgenic animals already are in clinical). The next step, at least for the industry leaders, is simply raising the necessary herds or flocks to produce enough bioactive protein to supply the trials.

"PPL Therapeutics and Genzyme Transgenics Corporation are probably closest to beginning clinical trials," according to John Logan, vice president of research & development, Nextran (Princeton, NJ). Both have farms and small flocks or herds in place.

PPL Therapeutics (Edinburgh, Scotland) has one of the broadest programs involving transgenic animals for the production of human proteins. The company has collaborative agreements with Novo Nordisk (Bagsvaerd, Denmark), Bayer AG (Germany) and the Wyeth-Ayerst (Philadelphia, PA) division of American Home Products to produce therapeutic proteins in the milk of rabbits, sheep and cows. PPL holds a U.S. patent for its method of using the beta-lactoglobulin gene promoter to produce any protein in the milk of all species of transgenic livestock and the recovery of the protein from the milk. A flock of sheep is well-established in Scotland, but PPL's first bull was born in October 1995 in the U.S., so development of its cattle line is still at least two to three years away.

\$12M Facility

The company recently began operations at its new \$12-million pilot facility (see page 1) for the transgenic production, initially, of human alpha-1-antitrypsin (AAT) for the treatment of lung disorders.

"Following AAT, the next products PPL has scheduled for the clinic are recombinant activated Protein C (to treat deep-vein thrombosis) and recombinant fibrinogen (for use as a tissue sealant)," according to Andrew Carver, sales manager. "PPL, in collaboration with Seattle-based ZymoGenetics, a U.S.-based subsidiary of Novo Nordisk, has successfully produced founder transgenic sheep for both of these proteins. Analysis of their milk revealed fibrinogen expression exceeding 5g/l and Protein C expression exceeding 0.4g/l. In both cases the proteins were correctly processed and biologically active."

Protein C is a complex, highly processed human plasma protein involved in blood clotting. It only works locally, leaving the coagulation

system intact, and so has the potential to overtake other anticoagulants in treating myocardial infarction, thromboembolic stroke, pulmonary embolism and thrombosis. Protein C is not commercially available, regardless of manufacturing process.

Fibrinogen currently is produced using transgenic mice, and large-scale production is being planned. It is the active agent in the blood-clotting process and could be used in situations after trauma or surgery, as well as for a tissue sealant when sutures or staples are inappropriate. PPL also has produced bioactive Factor IX, a compound that is deficient in patients with Hemophilia B. Levels of 0.6g/l have been produced in the milk of transgenic mice. Purification processes for each of these proteins are being developed. "...based upon our existing AAT purification model," Carver said.

Another blood clotting agent, antithrombin III (ATIII), developed by Genzyme Transgenics Corp. (Framingham, MA), is expected to enter Phase I clinical trials in the second half of 1996. This protein is derived from the blood plasma of transgenic goats and, in preclinical trials, "it performed similarly to plasma-derived ATIII in a number of animal models," according to the company. "We believe transgenic ATIII will prove to be a superior, more economical protein than the plasma-derived product on the market today," said James A. Geraghty, Genzyme's president and CEO. Genzyme's goal is to expand the use of ATIII and, ultimately, to eliminate the need for plasma-based ATIII.

Cancer Therapy

Last month, Genzyme Transgenics announced that a goat carrying the gene for BR96, a proprietary monoclonal antibody developed by Bristol-Myers Squibb (Princeton, NJ), has been born at Genzyme Transgenics' production facility in Charlton, MA. It is the first founder transgenic goat to be born at the commercial production facility.

Genzyme Transgenics also reported that it has expressed the BR96 antibody in the milk of transgenic mice, which indicates, according to company officials, that the antibody can be produced successfully in transgenic goats. BR96-doxorubicin conjugate, is being developed by Bristol-Myers Squibb for potential cancer therapies. Genzyme Transgenics is collaborating with Bristol-Myers Squibb to develop transgenic goats that will produce BR96 in high volumes to assess feasibility and poten-

tial application for commercial production.

Perry Fell, Ph.D., director of molecular immunology at Bristol-Myers Squibb, confirmed that the transgenic BR96 expressed in the mouse test system retained its ability to bind to human tumor cells. Bristol-Myers Squibb is currently in Phase II clinical trials with BR96-doxorubicin.

"This achievement indicates that the transgenic antibody is properly folded and thus can fulfill its intended role as an antibody designed to deliver the drug doxorubicin to a variety of carcinomas," said Dr. Fell.

"We now have evidence that when the development of transgenic goats is completed, there will be a very good chance of economically producing a clinically effective molecule," added Harry Meade, Ph.D., vice president of transgenic research at Genzyme Transgenics.

The company also received notification from the European Patent Office in January 1996 of the intent to grant a European patent for the development of transgenic proteins. The patent covers the basic DNA vector sequence of expressing proteins in milk and the basic method used to produce therapeutic proteins in the milk of transgenic animals. Genzyme has similar patents pending in the U.S. and Japan.

This March, Pharming (Leiden, the Netherlands) produced the world's first samples of human lactoferrin from transgenic cow's milk. In terms of biological activity, it is indistinguishable from lactoferrin produced by humans. This natural antibiotic is about to enter preclinical tests, focusing upon the treatment or prevention of gastrointestinal infections in patients with low immunological resistance, including premature infants, chemotherapy and radiotherapy patients and AIDS and cancer patients. This lactoferrin was produced from offspring of the transgenic bull, Herman. (New lines of transgenic animals, which can produce higher ratios of lactoferrin to milk, have since joined the herd.) The trials are being undertaken as part of a joint venture with Verenigde Bedrijven Nutricia (Zoetermeer, The Netherlands), the European market leader in developing clinical nutrition products. Pharming also has produced human serum albumin for trauma treatment, human collagen for tissue repair and human lysozyme, which is used in combination with lactoferrin for the treatment and prevention of gastrointestinal infections.

Pharming partnered with AutoImmune (Lexington, MA) one year ago for the production of human collagen



Genzyme Transgenics Corp. derived a blood clotting agent, antithrombin III, from the blood plasma of transgenic goats. The product is expected to enter Phase I clinical trials in the second half of 1996.

for the treatment of rheumatoid arthritis. Pharming will produce the collagen, and AutoImmune will conduct the preclinical testing. According to Patricia Nelson, Ph.D., director of immunology at AutoImmune, "The partnership is trying to produce therapeutic proteins in quantities large enough for use in humans. A small herd could produce (the necessary) quantities." Currently, Nelson said, Pharming is producing up to 2 g/l of the collagen "...depending upon the level of the promoter used." The testing currently remains in preclinical trials. Pharming also is working with Collagen Corp. (Palo Alto, CA) to produce human collagen in transgenic mice.

Human Hemoglobin

Nextran (Princeton, NJ) is developing human hemoglobin in transgenic pigs at levels of about 40g/l. According to Logan, "Human and pig hemoglobin are very similar and very concentrated." For example, "One liter of pig blood contains more than 125 grams of natural hemoglobin. In transgenic pigs, about 35 percent of that hemoglobin will be human. The work is in preclinical studies. We envision this as a follow-on..." to the blood substitute work underway at the parent company, Baxter (Deerfield, IL).

Prion Disease

Companies using transgenic livestock go to great lengths to protect their animals from contracting scrapie, a spongiform encephalopathy of sheep that is similar to the disease dubbed "mad cow disease" by the British press. Firms, including PPL and Genzyme, tend to import their sheep or goats directly from New Zealand—one of the three nations that

is recognized as being free of scrapie—and have established their farms on land that had never been used for livestock production and have built entirely new facilities. Because the disease in sheep has both a genetic and an infectious component, the firms ensure that their herds are several miles from any other livestock. In a similarly protective action, "PPL's bovine program is performed exclusively at the Blacksburg (Virginia) facility," Carver said. Nextran avoids this concern because, "Pigs do not have a scrapie equivalent, although they can be infected experimentally," explained Logan.

Despite the scare in the U.K., scientists report that that no trace of bovine spongiform encephalopathy (BSE) is found in the milk of animals infected with the disease. Additionally, the incidence of BSE has dropped precipitously since the U.K. banned the practice of feeding protein supplements from ruminants to other ruminants in 1990.

"Among sheep, scrapie was reported as early as 1832 but of the current 82,000 U.S. sheep flocks, few are believed to be infected with scrapie. Since the first U.S. case in 1947, fewer than 1,500 flocks have been diagnosed with scrapie," according to Curtis Youngs, Ph.D., associate professor of animal science, Iowa State University (Ames, IA). Additionally, none of the 1.2 thousand cattle herds in the U.S. have ever been diagnosed with BSE.

Although transgenic technology was oversold in the mid-1980s, shortly after it was introduced, the field seems to have a solid future. "This is a very active area of research," Dr. Youngs said. "A lot of major pharmaceutical companies are involved with transgenic animal research, but they don't necessarily advertise that."

The use of transgenic mice is far ahead of that of transgenic animals from agricultural species, according to Youngs. The reasons are cost, efficiency and generation intervals. The gestation time for a mouse is 20 days, compared to 280 days for a cow so, in the time it takes a cow to deliver a calf four or five generations of mice can be born, he said. Additionally a cow takes about 13 months to reach sexual maturity versus 35 days for laboratory mice. In addition, the percentage of injected embryos that develop into transgenic animals is about five times greater for mice than for farm animal species, Youngs said.

Despite any time delays, using transgenic animals to produce human proteins seems to make financial sense. PPL indicates that, compared to traditional manufacturing technologies (often vat fermentation), the use of transgenics offers a low capital investment, low production costs and a large-volume production. The rationale is that building and maintaining a farm production unit, using standard dairy management techniques is much less expensive and more scalable than the alternative, and that the number of animals on that farm can be adjusted easily to meet demand.

According to Logan, however, "A substantial cost is purification." It, however, shouldn't eliminate the capital savings.

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